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AROMATASE INHIBITION IN BOYS WITH DELAYED PUBERTY:

EFFECTS ON GROWTH, MATURATION, BONE, SERUM LIPIDS, AND INSULIN

by

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ACADEMIC DISSERTATION

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ABSTRACT

Estrogens have an essential role in the regulation of bone maturation in both sexes. Without the action of estrogens, growth plates remain open, allowing growth to continue even in adulthood. Some boys with constitutional delay of puberty (CDP) are unable to fully exploit their genetic growth potential. This prospective, randomized, placebo-controlled study was undertaken to evaluate whether suppression of estrogen synthesis in boys with CDP delays bone maturation and ultimately results in increased adult height.

A total of 23 boys with CDP received a conventional, low-dose testosterone treatment for 6 months to induce the progression of puberty. Eleven of these boys were randomized to receive a specific and potent P450 aromatase inhibitor, letrozole, for one year, to suppress estrogen production, and 12 boys were randomized to receive placebo for one year. Another 10 boys received no treatment, thus serving as the untreated group.

Letrozole was well tolerated, and no side-effects sufficient to indicate discontinuation of the treatment were observed. Letrozole inhibited estrogen synthesis effectively. During treatment with testosterone and letrozole 17β-estradiol concentrations remained at the pretreatment level, while they increased during treatment with testosterone alone as well as during spontaneous progression of puberty. Testosterone concentrations increased in all groups, but during letrozole treatment the increase was more than 5-fold higher than in the group treated with testosterone alone. Letrozole treatment delayed bone maturation. During the 18-month follow-up bone age advanced 0.9 year in the group treated with testosterone and letrozole and 1.7 years in the group treated with testosterone alone. From start to 18 months, an increase of 5.1 cm in predicted adult height was seen in the boys receiving testosterone and letrozole, while no change was seen in those receiving testosterone alone or in the untreated boys. This finding suggests that adult height can be increased in growing adolescents by suppressing estrogen synthesis. Furthermore, the boys with CDP may achieve an adult height closer to their genetic growth potential if estrogen actions are inhibited.

No differences were present in changes in bone mineral content, bone mineral density (BMD), or bone mineral apparent density (BMAD), an estimate of true volumetric BMD, between the two treated groups, suggesting that a one-year treatment with letrozole is unlikely to have any major harmful effects on developing peak bone mass in pubertal boys. However, although lumbar spine BMAD increased in both treated groups, in the group treated with testosterone and letrozole, the increase was

statistically significant only 6 months after discontinuation of letrozole treatment. Thus, close follow-up of BMD during treatment with P450 aromatase inhibitors is warranted.

The HDL-cholesterol concentration decreased in the group treated with testosterone and letrozole, whereas no change was observed in the group treated with testosterone alone. However, 6 months after the discontinuation of all treatments, the HDL-cholesterol concentrations were similar in all groups. The concentrations of LDL-cholesterol or triglycerides did not change in any of the groups. Treatment with P450 aromatase inhibitors may have disadvantageous effects on HDL-cholesterol, and therefore, follow-up of the lipid profile is important.

Insulin concentrations decreased during letrozole treatment, while no change was observed during treatment with testosterone alone. Thus, inhibition of estrogen synthesis in early and midpubertal boys does not appear to diminish insulin sensitivity. As the boys on letrozole treatment had a higher increase in androgen concentrations, the finding further suggests that rising androgen concentrations during puberty do not directly contribute to the development of puberty-associated insulin resistance in boys.

This study also clarified the role of endogenous estrogens in the regulation of gonadotropin secretion. We demonstrated that low physiological concentrations of endogenous estrogens inhibit LH and FSH secretion in boys during early and midpuberty. Moreover, during this stage of puberty the endogenous estrogen-mediated inhibition of LH secretion appears to occur at the site of the pituitary.

In conclusion, P450 aromatase inhibitor treatment in boys with delayed puberty delays bone maturation and increases predicted adult height. This suggests that adult height can be increased in growing adolescents by suppressing estrogen synthesis. Future studies are needed to establish whether fourth-generation P450 aromatase inhibitors can effectively treat various growth disorders.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals I-IV:

I Wickman S, Sipilä I, Ankarberg-Lindgren C, Norjavaara E, Dunkel L. A specific aromatase inhibitor and potential increase in adult height in boys with delayed puberty: a randomised controlled trial. Lancet 357:1743-1748, 2001.

II Wickman S, Dunkel L. Inhibition of P450 aromatase enhances gonadotropin secretion in early and midpubertal boys: evidence for a pituitary site of action of endogenous E. J Clin Endocrinol Metab 86:4887-4894, 2001.

III Wickman S, Saukkonen T, Dunkel L. The role of sex steroids in the regulation of insulin sensitivity and serum lipid concentrations during male puberty: a prospective study with a P450-aromatase inhibitor. Eur J Endocrinol 146:339-346, 2002.

IV Wickman S, Kajantie E, Dunkel L. Effects of suppression of estrogen action by the P450 aromatase inhibitor letrozole on bone mineral density and bone turnover in pubertal boys. J Clin Endocrinol Metab 88:3785-3793, 2003.

ABBREVIATIONS

ALP	Alkaline phosphatase
BMAD	Bone mineral apparent density
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
CDP	Constitutional delay of puberty
CTx	Cross-linked carboxyterminal telopeptide of type I collagen
CV	Coefficient of variation
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
ER	Estrogen receptor
FFM	Fat-free mass
FM	Fat mass
FSH	Follicle-stimulating hormone
G	Genital stage according to Tanner
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
HDL	High-density lipoprotein
ICTP	Cross-linked carboxyterminal telopeptide of type I collagen
IGF	Insulin-like growth factor
IGFBP	IGF-binding protein
LDL	Low-density lipoprotein
LH	Luteinizing hormone
OC	Osteocalcin
P	Pubic hair stage according to Tanner
p	Probability
PICP	Carboxyterminal propeptide of type I procollagen
r	Correlation coefficient
RIA	Radioimmunoassay
SD	Standard deviation
SEM	Standard error of the mean

INTRODUCTION

Increasing sex steroid secretion during puberty induces acceleration of growth and development of secondary sexual characteristics. Androgens in boys and estrogens in girls have been generally assumed to be the primary sex steroids causing the physical changes during puberty.

In 1994, the description of the estrogen receptor (ER) α-negative male revolutionized the traditional concept of the roles of sex steroids in the male (Smith et al. 1994). This 28-year-old man was 204 cm tall, had open epiphyses of long bones and consequently was still growing. He had no recollection of accelerated pubertal growth despite otherwise normal pubertal virilization. Soon thereafter, two males with similar phenotypes were described (Morishima et al. 1995; Carani et al. 1997). In these men, the effects of estrogens were suppressed due to mutations in the gene coding the P450 aromatase enzyme which converts androgens to estrogens. Estrogen administration in these men closed the epiphyses and discontinued growth (Carani et al. 1997; Bilezikian et al. 1998). In all of these men, concentrations of androgens were normal or above normal. These case reports confirm that estrogens are essential for epiphyseal closure in males. Moreover, the reports suggest that estrogens induce pubertal growth acceleration but do not participate significantly in the regulation of linear prepubertal growth, since in the absence of estrogen action, growth progresses with a steady velocity.

Delayed puberty is defined as a lack of initial signs of puberty by an age that is more than 2 standard deviation (SD) above the mean for the population. In most instances, the delay in pubertal development is not due to any underlying illness. Therefore, it can be regarded as an extreme end of the normal spectrum of pubertal timing and is defined as constitutional delay of puberty (CDP). However, some boys with CDP do not fully exploit their genetic growth potential, i.e., they remain shorter as adults than predicted from their parents' heights (Crowne et al. 1990; LaFranchi et al. 1991; Albanese et al. 1993, 1995). The delay in puberty and growth can result in considerable psychological distress for an adolescent, and in these situations, medical intervention is justified. These boys have been treated with androgens or anabolic steroids to induce development of secondary sexual characteristics and growth acceleration (Martin et al. 1986; Richman et al. 1988; Kaplowitz 1989; Albanese et al. 1994). However, these treatments do not increase adult height (Blethen et al. 1984; Martin et al. 1986; Albanese et al. 1993).

The role of estrogens in the regulation of bone maturation is unequivocal. The purpose of this study was explore whether inhibition of estrogen synthesis in boys with CDP delays maturation of growth plates and ultimately results in increased adult height.

This treatment may provide a means to help these boys achieve an adult height closer to their genetic growth potential.

REVIEW OF THE LITERATURE

MALE PUBERTY

Hypothalamic-pituitary-testicular axis

Hypothalamus and pituitary

The hypothalamic-pituitary-testicular axis is already operative in the prenatal period. High circulating gonadotropin concentrations at midgestation decrease before birth. During the first months of life the hypothalamic-pituitary-testicular axis is reactivated; the concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), sex steroids, and inhibin B are higher than in older prepubertal children (Forest et al. 1974; Waldhauser et al. 1981; Andersson et al. 1998; Byrd et al. 1998; Bergadá et al. 1999). The concentrations decrease early and remain relatively low in prepubertal years (Forest et al. 1974; Wu et al. 1989; Dunkel et al. 1990a, 1992; Wu et al. 1991, 1996; Byrd et al. 1998; Bergadá et al. 1999).

The increase of gonadotropin-releasing hormone (GnRH) secretion in the hypothalamus, and consequently, gonadotropin secretion in the pituitary marks the onset of puberty. The current view is that the onset of puberty results from the removal of a central mechanism that inhibits GnRH secretion in prepuberty (Plant 2001). Augmentation of gonadotropin secretion first occurs at night (synchronized with sleep), leading to the clear night-day difference in gonadotropin concentrations (Boyar et al. 1972, 1974; Parker et al. 1975; Dunkel et al. 1990a, 1992; Oerter et al. 1990; Wu et al. 1991, 1996). With advancing puberty, daytime secretion also increases, with disappearance of the day-night difference at late puberty (Boyar et al. 1972, 1974; Parker et al. 1975; Oerter et al. 1990; Wu et al. 1996). GnRH and gonadotropins are secreted episodically (Waldhauser et al. 1981; Dunkel et al. 1990a, 1992; Wu et al. 1991, 1996). The increase in LH and FSH concentrations during puberty is mainly due to an increase in gonadotropin pulse amplitude with a smaller increase in pulse frequency (Wu et al. 1996).

The role of LH in the testes is to stimulate Leydig cells to secrete sex steroids, mainly testosterone. FSH controls the function of Sertoli cells, including secretion of inhibin B, and regulates spermatogenesis. During puberty sex steroid concentrations gradually increase, reaching the adult range at late puberty (Manasco et al. 1995; Klein et al. 1996; Byrd et al. 1998; Mitamura et al. 1999). Sex steroid concentrations are higher during sleep, and peak concentrations occur in the morning (Boyar et al. 1974;

Parker et al. 1975; Veldhuis et al. 1984; Mitamura et al. 1999). Inhibin B concentration increases at early puberty (Andersson et al. 1997; Byrd et al. 1998; Raivio et al. 1998; Crofton et al. 2002), reaching an adult male level at pubertal stage II (Andersson et al. 1997).

The testicular hormones also participate in the control of gonadotropin secretion by a feedback mechanism at the hypothalamus and the pituitary gland. In girls with primary gonadal failure, LH and FSH concentrations are above normal during infancy, normal or near-normal during childhood, and supranormal after the age of the onset of puberty (Conte et al. 1975). In prepubertal boys with primary testicular failure, FSH concentrations are above normal, but LH concentrations usually remain within normal range (Dunkel et al. 1990b). These findings suggest that testicular hormones are involved in negative feedback regulation of gonadotropin secretion during infancy (Conte et al. 1975). In childhood, testicular hormones have a role at least in the regulation of FSH concentrations (Dunkel et al. 1990b), although gonadotropin secretion is primarily under the control of the central nervous system (Plant 2001). After the onset of puberty, testicular hormones negatively regulate gonadotropin secretion (Conte et al. 1975). During puberty the mechanism of sex steroid-mediated inhibition changes. In early and midpubertal boys, testosterone infusion decreases LH concentrations and pulse frequency (Kletter et al. 1994), which is assumed to reflect the frequency of hypothalamic GnRH secretion pulses (Clarke et al. 1982; Levine et al. 1982). However, it has no effect on LH pulse amplitude or GnRH-induced LH release (Kletter et al. 1994). These findings indicate that testosterone suppresses gonadotropin secretion primarily at the site of the hypothalamus in boys during early and midpuberty. In adult men, testosterone infusion decreases LH concentrations, LH pulse amplitude, and GnRH-induced LH release, but has no effect on LH pulse frequency, suggesting the pituitary as the primary site of negative feedback action of testosterone (Kletter et al. 1992). Testosterone administration decreases gonadotropin concentrations both in normal men and in men with idiopathic hypogonadotropic hypogonadism whose pituitary-gonadal function has been normalized with long-term pulsatile GnRH replacement, but a greater decrease is observed in normal men (Finkelstein et al. 1991). This indicates both pituitary and hypothalamic effects of testosterone (Finkelstein et al. 1991). Thus, sex steroids appear to inhibit gonadotropin secretion at the hypothalamus from early puberty onwards. The available evidence suggests that during puberty sensitivity of the pituitary gland to sex steroid-mediated negative feedback increases such that in adult men sex steroids inhibit gonadotropin secretion also at the site of the pituitary.

Testis

Androgens

Androgens are metabolized from cholesterol. The major potent circulating androgen in men is testosterone, which mostly circulates bound to plasma proteins, with approximately 2% occurring in free form (Dunn et al. 1981). More than 95% of testosterone is secreted from the testes (Riggs et al. 2002). Testosterone can be converted by the enzyme 5α-reductase to 5α-dihydrotestosterone (DHT), the majority of which is formed in extragonadal sites (Riggs et al. 2002; Figure 1). DHT is the main source of androgenic activity in many target tissues (Riggs et al. 2002). The adrenal cortex and the testis also secrete other androgens, mainly dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA-S), and Δ^4-androstenedione. These hormones have only weak androgenic activity, but they are important substrates for extragonadal synthesis of potent sex steroids (Riggs et al. 2002; Figure 1).

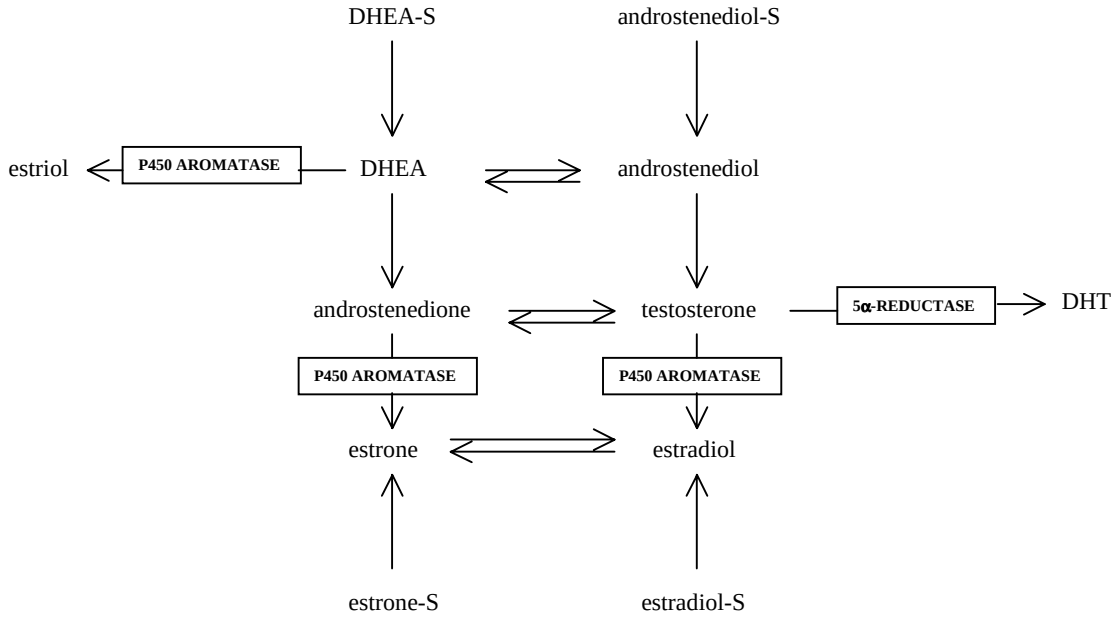

Figure 1. Main pathways for extragonadal synthesis of sex steroids. Reversible (double arrows) and irreversible (single arrows) reactions are indicated. DHT, dihydrotestosterone; DHEA, dehydroepiandrosterone; S, sulfate.

Estrogens and P450 aromatase enzyme

P450 aromatase is the key enzyme for estrogen biosynthesis. It catalyzes conversion of androgens to estrogens (Figure 1). P450 aromatase is encoded by a single gene, the *CYP19*, which is localized on chromosome 15q21.2. The same enzyme is expressed in many tissues, e.g. the testis, ovary, placenta, adipose tissue, bone, and brain (Simpson et al. 1994). Its expression is controlled by tissue-specific promoters regulated by different transcription factors (Simpson et al. 1994, 2001). Via P450 aromatase, testosterone is converted to estradiol, androstenedione to estrone, and DHEA to estriol. In males, the majority of estradiol and estrone are formed at extragonadal sites (MacDonald et al. 1979). Although the circulating amounts of estrogens in males are relatively low, the local concentrations at those tissue sites where P450 aromatase is expressed are probably higher (Labrie et al. 1997, 1998; Simpson et al. 2001). Thus, local paracrine and intracrine actions of estrogens may have an important physiological role (Labrie et al. 1997, 1998).

Inhibin B

Inhibin B is a glycoprotein hormone, a dimer consisting of an α-subunit and a βB-subunit. In males, it is produced mainly by the testis. Inhibin B has a physiological role in the feedback control of FSH secretion and is likely to be the most important feedback regulator of FSH secretion in adult males (Nachtigall et al. 1996; Anderson et al. 1997; Hayes et al. 2001). Concentrations of inhibin B and FSH correlate negatively from early to midpuberty onwards, suggesting that the negative feedback regulation loop maturates during puberty (Andersson et al. 1997; Byrd et al. 1998; Raivio et al. 2000).

Normal physical development

Rising concentrations of sex steroids during puberty induce development of secondary sexual characteristics and an increase in growth rate. The first sign of sexual maturation in boys is enlargement of testes and attainment of genitalia stage (G; according to Tanner) 2, which normally occurs between the ages of 9.5 and 13.5 years (Tanner 1962). This is followed by a gradual increase in penile size and appearance of pubic hair. Axillary hair and the characteristic body odor appear, and voice pitch is lowered. An increase in height velocity starts after Tanner stage G 3, and the peak growth spurt (about 9.5 cm/year) occurs between stages G 3 and 5 (Tanner 1962; Tanner et al. 1966). The pubertal growth spurt lasts approximately 2 years, after which growth rate abruptly decelerates and eventually stops with the closure of epiphyseal growth plates (Tanner et al. 1966, 1985). The apex of increase in muscle mass is a late event occurring after the

peak growth spurt (Tanner 1962). Chest and facial hair appear at late puberty. Spermarche, the onset of the release of spermatozoa, usually occurs during the early stages of puberty, but considerable variation in pubertal characteristics is present at that time (Nielsen et al. 1986).

In addition to wide variation at the age of onset of puberty, the time spent at any given stage of puberty and the order of appearance of different events also varies (Marshall et al. 1970; Nielsen et al. 1986). Boys normally progress from Tanner stage G 2 to 5 in about 3 years, although some boys take longer than 5 years (Marshall et al. 1970).

Constitutional delay of puberty

About 2.5% of children are delayed in developing secondary sexual characteristics. In most cases, there is no underlying pathology, and the condition represents an extreme end of the normal spectrum of pubertal timing. This developmental pattern is defined as constitutional delay of puberty (CDP) or constitutional delay of growth and puberty/maturation/development. CDP tends to be familial, although not all of the boys have positive family histories (Sedlmeyer et al. 2002). Characteristic for CDP is that stature is short for chronological age but appropriate for bone age and stage of pubertal development. Bone age is typically delayed to the same extent as puberty. Once puberty begins, it advances at normal rates. However, if puberty is much delayed, both peak height velocity and duration of growth spurt are smaller, and consequently, the total pubertal height gain is reduced. During late prepuberty and early puberty most of the height gain takes place in the extremities, and during the pubertal growth spurt in the spine. Thus, some individuals with CDP may end up with body disproportion at final adult height, i.e., a short spine in relation to leg length (Crowne et al. 1990; Albanese et al. 1993, 1995). Many studies also suggest that some boys with CDP do not exploit their full genetic growth potential (Crowne et al. 1990; LaFranchi et al. 1991; Albanese et al. 1993, 1995). Furthermore, men with a history of CDP have been observed to have osteopenia in adult life (Finkelstein et al. 1992, 1996, 1999), but volumetric bone mineral density (BMD) has also been demonstrated to be normal in these men (Bertelloni et al. 1998a).

Boys with CDP are smaller in height and in muscle mass and far less developed in secondary sexual characteristics compared with their age-matched peers. The delay in puberty and growth can lead to considerable psychological distress, and in these situations, medical intervention is justified. These boys have been treated with low-dose androgens or anabolic steroids to induce an increase in growth velocity and

development of secondary sexual characteristics (Martin et al. 1986; Richman et al. 1988; Kaplowitz 1989; Albanese et al. 1994). Although capable of accelerating growth velocity, these treatments do not increase adult height (Blethen et al. 1984; Martin et al. 1986; Albanese et al. 1993).

SPECIFIC ROLES OF ANDROGENS AND ESTROGENS IN MALES

Growth

Growth velocity decreases during early childhood from the rapid rate of the first months of life, reaching a nadir (about 5 cm/year) just at the onset of puberty or in the early stages of puberty (Tanner et al. 1966, 1985). Growth velocity accelerates again at midpuberty, after the genital stage G 3 (at the age of 10.5 to 16 years), and reaches the highest value, about 9.5 cm/year, during mid- to late puberty, between the Tanner stages of G 3 and 5 (Tanner 1962; Tanner et al. 1966). Growth then decelerates and, at about the age of 18 years, stops with the closure of the epiphyses (Tanner et al. 1966, 1985).

Growth is influenced by multiple regulators, including genetic, hormonal (e.g. growth hormone (GH), estrogens, androgens, thyroid hormones, glucocorticoids, insulin), and environmental (e.g. nutrition, emotional and physical well-being) factors.

Androgens

Testosterone has clearly been shown to stimulate growth. Testosterone concentrations correlated positively with growth velocity in adolescent boys (Merimee et al. 1991; Adan et al. 1994), testosterone infusion stimulated ulnar growth in pre- and early pubertal boys (Cassorla et al. 1984), and testosterone treatment in boys with CDP accelerated growth (Richman et al. 1988; Kaplowitz 1989; Albanese et al. 1994; Crowne et al. 1995). Since testosterone is able to be aromatized to estradiol or reduced to DHT, its growth-promoting effects may be androgen- or estrogen-mediated. An androgen-mediated mechanism is suggested by the finding that a nonaromatizable androgen, DHT, induced ulnar growth in pre- and early pubertal boys (Cassorla et al. 1984). Although exogenous androgens stimulate growth in boys, endogenous androgens do not appear to have a very important role in regulation of the pubertal growth spurt, since in patients with complete androgen insensitivity syndrome (genetic males,

karyotype 46XY) with intact gonads, pubertal growth velocity is similar to that in normal females, albeit lower than in normal males (Zachmann et al. 1986).

Androgen receptors have been localized in human growth plate chondrocytes (Abu et al. 1997), suggesting that androgens may stimulate growth by a direct local effect. It is unclear whether androgens have an influence on GH secretion. In one study, a nonaromatizable anabolic steroid, oxandrolone, increased GH secretion in pre- and early pubertal boys (Ulloa-Aguirre et al. 1990), but in another study, GH and insulin-like growth factor I (IGF-I) concentrations did not change during oxandrolone treatment (Link et al. 1986). Moreover, DHT, a nonaromatizable androgen, decreased (Keenan et al. 1993) or did not change (Eakman et al. 1996) GH concentration in pre- and early pubertal boys, which suggests that growth stimulating effects of androgens are not mediated through the GH-IGF-I system.

Estrogens

A low-dose estradiol infusion accelerated ulnar growth in pre- and early pubertal boys, but higher doses had no effect on ulnar growth (Caruso-Nicoletti et al. 1985). In boys with familial male-limited precocious puberty, an aromatase inhibitor, testolactone, decreased growth velocity to near- normal prepubertal values, while an antiandrogen, spironolactone, alone did not decrease growth velocity significantly until testolactone was added (Laue et al. 1989). This suggests that estrogens are more important than androgens in pubertal growth acceleration in boys (Laue et al. 1989). The essential role of estrogens in the regulation of male growth was confirmed when a man with an inactivating mutation in the ERα gene was described (Smith et al. 1994). This 28-year-old man was 204 cm tall (Smith et al. 1994). He had a bone age of 15 years, thus, the epiphyses of long bones were open and he was still growing (Smith et al. 1994). He had no recollection of pubertal growth acceleration (Smith et al. 1994). Soon thereafter, two other adult males with suppression of estrogen action due to mutations in the gene coding the P450 aromatase enzyme were described (Morishima et al. 1995; Carani et al. 1997). At the time of diagnosis, these individuals were tall, had markedly delayed bone ages, and were still growing (Morishima et al. 1995; Carani et al. 1997). In these two aromatase-deficient men, estrogen administration closed the epiphyses and discontinued growth (Carani et al. 1997; Bilezikian et al. 1998). In all of these men, androgen concentrations were normal or above normal (Smith et al. 1994; Morishima et al. 1995; Carani et al. 1997; Bilezikian et al. 1998). These unique case reports suggest that endogenous estrogens are needed to induce the male pubertal growth spurt, but linear growth occurs without the action of estrogens. Furthermore, they clearly prove that estrogen action is needed for the closure of epiphyses and cessation of growth. A longitudinal follow-up of growth velocity and hormonal parameters in boys during

puberty provided further information about the role of estrogens in regulation of male pubertal growth (Klein et al. 1996). The first significant increase in estrogen concentration, determined by an ultrasensitive bioassay, occurred simultaneously with the peak growth velocity (Klein et al. 1996). However, at the time of the highest growth velocity, estrogen concentrations were still relatively low (Klein et al. 1996). Estrogen concentrations correlated positively with growth velocity before the peak growth spurt and negatively thereafter (Klein et al. 1996). All of these findings are concordant with the concept that the effect of estrogens on epiphyseal growth is biphasic (Figure 2). Low levels of estrogens, typical for early and midpubertal boys, stimulate growth, and higher levels, typical for late pubertal males, do not appear to be optimal for growth but instead accelerate epiphyseal fusion.

ERα and ERβ have been localized in growth plate chondrocytes in humans, suggesting that estrogens are able to act directly at the epiphyseal growth plate (Kusec et al. 1998; Nilsson et al. 1999; Egerbacher et al. 2002). ER blockade with tamoxifen decreased GH secretion and IGF-I concentrations in late pubertal boys, indicating a stimulating effect of endogenous estrogens on GH secretion (Metzger et al. 1994). Although low-dose estrogen administration has been shown to increase GH production rate (Mauras et al. 1990), high-dose estrogen decreases IGF-I concentrations (Copeland 1988). Thus, the biphasic effect of estrogens on growth can also partly be mediated through the GH-IGF-I system (Figure 2).

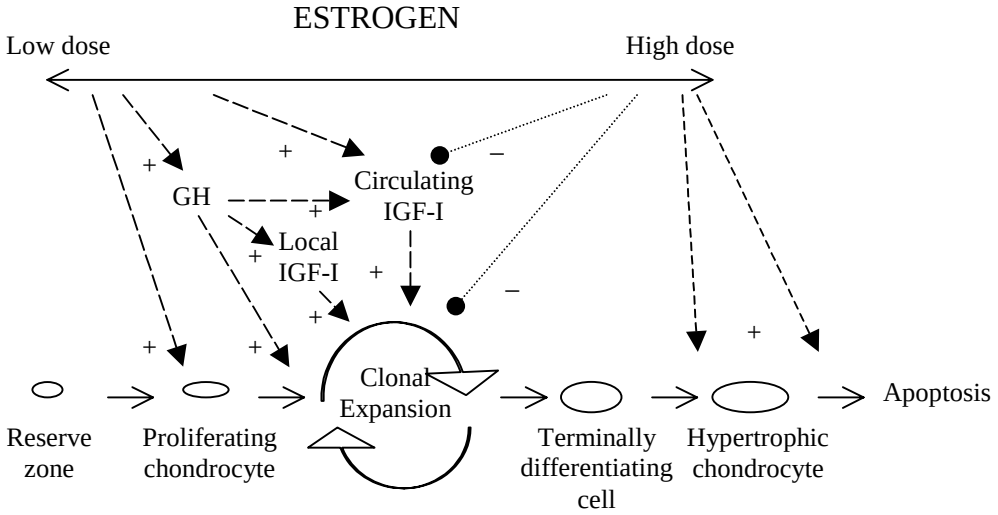

Figure 2. Suggested model of the effects of estrogen in epiphyseal growth plate. GH, growth hormone; IGF-I, insulin-like growth factor I; +, enhancing effect; –, suppressing effect.

Bone mineral density and bone turnover

Bone mass increases throughout childhood, with the maximum increment rate occurring during puberty, when bone mass doubles (Gilsanz et al. 1988; Bonjour et al. 1991; Kröger et al. 1993; Faulkner et al. 1996). The increase in bone mass results chiefly from growth in bone size; an increase in volumetric BMD has a smaller impact (Kröger et al. 1992, 1993; Faulkner et al. 1996). Bone size increases by growth in length and by modeling, i.e., growth in thickness. Bone elongation ends with the closure of epiphyses around the age of 18 years in boys (Tanner et al. 1966, 1985), and bone modeling ends around the age of 20 years, approximately when peak bone mass is reached (Bonjour et al. 1991; Kröger et al. 1993; Välimäki et al. 1994). Simultaneously with bone growth, old bone is continuously replaced by new bone. This coupled process of bone resorption and formation is called remodeling. Thus, during growth the rate of bone turnover is affected by three different biological processes, i.e., longitudinal growth, modeling, and remodeling.

Sex steroids have an important role in bone growth and in the development of peak bone mass. Hypogonadism has deleterious effects on peak bone mass (Finkelstein et al. 1987). Furthermore, sex steroids are responsible for sexual dimorphism of the skeleton, i.e. larger bones with larger diameter and greater cortical thickness in men than in women (Vanderschueren et al. 1998).

Androgens

Patients with androgen insensitivity syndrome have decreased BMD, even before the age when peak bone mass is reached (Bertelloni et al. 1998b; Marcus et al. 2000). This indicates that androgens have an essential role in the development of peak bone mass. The important role of androgens in bone mass accretion is further supported by men having larger bones with a thicker cortex than women. Indeed, in male rats, periosteal bone formation was stimulated by testosterone and DHT (Turner et al. 1990). Testosterone was also associated positively, and independently of estrogen, with BMD in adult men (Khosla et al. 1998; Center et al. 1999; van den Beld et al. 2000).

The mechanism underlying regulation of bone metabolism by sex steroids was investigated in a study in elderly men whose gonadotropin secretion was suppressed by a GnRH agonist and conversion of androgens to estrogens was blocked by a P450 aromatase inhibitor and simultaneously estradiol alone, testosterone alone, both, or neither were administered (Falahati-Nini et al. 2000). During different interventions serum concentrations of bone turnover markers were determined, with results indicating that testosterone, probably via an androgen-mediated mechanism, maintains bone formation, while its role in bone resorption is smaller (Falahati-Nini et al. 2000).

Estrogens

The young adult men who cannot respond to or produce estrogens due to a defective ERα or P450 aromatase enzyme had significantly reduced BMD despite normal or supranormal androgen concentrations; estrogen administration improved BMD in the men with aromatase deficiency, whereas testosterone was ineffective (Smith et al. 1994; Morishima et al. 1995; Carani et al. 1997; Bilezikian et al. 1998). These case reports confirm the essential role of estrogens in the regulation of peak bone mass development in males. The correlation of bioavailable estradiol concentrations with BMD of the arms in pre- and early pubertal boys (Klein et al. 1998), and the relationship between an ER gene polymorphism and BMD in late pubertal boys (Lorentzon et al. 1999) are in accord with the concept of an important role for estrogens in bone mass accretion during growth and maturation in boys. The rate of increase in BMD of the arms in young men correlated positively with the concentrations of estrogen but not with those of testosterone (Khosla et al. 2001), which suggests that estrogens are more important than androgens in peak bone mass accretion in males. After attainment of peak bone mass, estrogen appears to be the dominant sex steroid for maintaining bone mass. Estrogen concentration, rather than that of testosterone, related positively with BMD in multivariate analysis in adult men (Slemenda et al. 1997; Khosla et al. 1998; Ongphiphadhanakul et al. 1998; Szulc et al. 2001), and in elderly men, the rate of bone loss associated better with the concentrations of bioavailable estradiol than with those of testosterone (Khosla et al. 2001).

Estrogens appear to regulate bone turnover by decreasing bone resorption and increasing bone formation which was demonstrated in the above-mentioned direct interventional study, in which gonadotropin secretion and activity of P450 aromatase enzyme were suppressed before the administration of estradiol alone, testosterone alone, both, or neither in elderly men (Falahati-Nini et al. 2000). Furthermore, suppression of estrogen synthesis by a P450 aromatase inhibitor, anastrozole, in elderly men was accompanied by an increase in bone resorption marker and a decrease in bone formation markers (Taxel et al. 2001).

Serum lipid concentrations

Serum lipid concentrations change during adolescence simultaneously with the changes in body composition and the concentrations of several hormones. In boys, high-density lipoprotein (HDL) cholesterol concentration decreases and concentration of triglycerides increases (Morrison et al. 1978, 1979; Laskarzewski et al. 1983a; Viikari et

al. 1985; Porkka et al. 1994). An increase in low-density lipoprotein (LDL) cholesterol concentration appears to occur at late puberty (Morrison et al. 1979). Sex hormones are suggested to have a role in the regulation of lipid concentrations during puberty since sex differences in lipid profiles develop during adolescence, leading to a more atherogenic lipid profile in men than in women (Heiss et al. 1980).

Androgens

In adult men under physiological conditions, androgens do not appear to have disadvantageous effects on lipid concentrations. Higher HDL-cholesterol concentrations have been found to be associated with increased testosterone (Gutai et al. 1981; Heller et al. 1983; Dai et al. 1984; Lichtenstein et al. 1987; Haffner et al. 1993) or DHT (Hämäläinen et al. 1986) concentrations, after taking into account factors affecting lipid metabolism. No relationship between concentrations of LDL-cholesterol and testosterone (Heller et al. 1983; Dai et al. 1984; Haffner et al. 1993) or DHT (Hämäläinen et al. 1986) has been observed. Concentrations of triglycerides have been demonstrated to have no association (Heller et al. 1983; Dai et al. 1984) or an inverse association (Lichtenstein et al. 1987; Haffner et al. 1993) with testosterone concentrations.

The relationship between androgens and lipids in boys during puberty differs from that of adult males. In adolescent boys, higher testosterone concentrations were associated with lower HDL-cholesterol concentrations even when factors affecting lipid metabolism had been taken into account (Morrison et al. 1998, 2000). Moreover, testosterone (Kirkland et al. 1987; Arslanian et al. 1997) or DHT (Saad et al. 2001) treatment in boys with delayed puberty decreased HDL-cholesterol concentration. These findings suggest that HDL-cholesterol is regulated via an androgen-mediated mechanism in pubertal boys. Consequently, rising concentrations of androgens during puberty may induce a puberty-associated decrease in HDL-cholesterol.

The role of androgens in the regulation of LDL-cholesterol in boys during puberty is unclear. In boys with delayed puberty, testosterone treatment was associated with a decreasing trend in LDL-cholesterol concentration, which did not, however, reach statistical significance (Arslanian et al. 1997), and DHT treatment had no effect on LDL-cholesterol concentration (Saad et al. 2001). An inverse relationship between testosterone and LDL-cholesterol concentrations was observed in one cross-sectional study (Morrison et al. 1998), but no association was found in another study (Morrison et al. 2000). Thus, androgens may not play as significant a role in the regulation of LDL-cholesterol as in the regulation of HDL -cholesterol in adolescent boys.

Androgens do not appear to significantly influence the regulation of triglycerides in boys during puberty. Testosterone and DHT treatment in boys with delayed puberty

had no impact on the concentrations of triglycerides (Arslanian et al. 1997; Saad et al. 2001). Nor was there any association between concentrations of testosterone and triglycerides in adolescent boys (Morrison et al. 1998, 2000).

In conclusion, the role of androgens in the puberty-associated changes in lipid concentrations remains somewhat unclear. While evidence suggests that in boys during puberty androgens contribute to the puberty-associated decrease in HDL-cholesterol concentrations, their role in the regulation of LDL-cholesterol and triglycerides may be less important.

Estrogens

Men with suppressed estrogen action due to mutations in P450 aromatase had an atherogenic lipid profile, i.e., subnormal HDL-cholesterol concentrations and elevated concentrations of LDL-cholesterol and triglycerides (Morishima et al. 1995; Carani et al. 1997). Estrogen administration in these men increased HDL-cholesterol concentration and decreased concentrations of LDL-cholesterol and triglycerides (Carani et al. 1997; Bilezikian et al. 1998). Low-dose estradiol administration in elderly men increased HDL-cholesterol concentration and decreased concentrations of LDL-cholesterol and triglycerides (Giri et al. 1998). Thus, estrogens at physiological levels appear to affect lipid concentrations in adult males favourably.

In adolescent boys, however, no relationship between estradiol and HDL-cholesterol concentrations has been observed (Morrison et al. 1998, 2000). Estradiol concentrations were not associated with the concentrations of LDL-cholesterol or triglycerides in one study (Morrison et al. 1998), while an inverse relationship was shown in another study (Morrison et al. 2000). By contrast, changes in lipid concentrations during male adolescence have been shown to be explained to some extent by changes in estradiol concentration, as well as by changes in testosterone concentration and body mass (Laskarzewski et al. 1983a, 1983b). Evidence for the favorable effect of estrogens at physiological concentrations on lipids in adult males, and the contradictory results obtained in studies of adolescent boys may be related to the roles of sex steroids in the regulation of lipid metabolism varying in different age groups (Srinivasan et al. 1986).

Insulin sensitivity

During puberty insulin action is impaired and insulin secretion is increased in both sexes (Amiel et al. 1986, 1991; Bloch et al. 1987; Caprio et al. 1989; Smith et al. 1989); with the completion of pubertal development, insulin resistance subsides (Amiel et al.

1986; Caprio et al. 1989; Smith et al. 1989). The decrease in insulin sensitivity during puberty appears to be restricted to peripheral glucose metabolism, and compensatory hyperinsulinemia may thus facilitate protein anabolism during rapid growth by amplifying insulin's effect on amino acid metabolism (Amiel et al. 1991; Heptulla et al. 1997). Insulin sensitivity decreases during puberty simultaneously with rising concentrations of sex steroids (Goran et al. 2001). However, whether sex steroids regulate insulin sensitivity in males during puberty is unclear.

A large body of evidence suggests that the decrease in insulin sensitivity during puberty is due to increasing action of GH. In studies including both sexes, insulin sensitivity correlated negatively with concentrations of GH (Amiel et al. 1986) and IGF-I (Bloch et al. 1987; Caprio et al. 1989; Smith et al. 1989), and insulin sensitivity decreased and insulin secretion increased during GH treatment in children with short stature (Heptulla et al. 1997). However, relationships between GH and insulin sensitivity in studies including only boys have been contradictory. In one longitudinal study and in several cross-sectional studies of adolescent boys, insulin sensitivity, measured by an intravenous glucose tolerance test, was not associated with parameters reflecting GH secretion (Cook et al. 1993; Travers et al. 1995; Goran et al. 2001). Moreover, testosterone treatment of seven boys with delayed puberty did not alter insulin sensitivity, measured by the hyperinsulinemic-euglycemic clamp, despite an increase in IGF-I and mean nocturnal GH concentrations (Arslanian et al. 1997). However, in a recent study of 189 adolescent boys, IGF-I and IGF-binding protein (IGFBP) 3 concentrations were correlated strongly with insulin resistance, determined by the hyperinsulinemic-euglycemic clamp, suggesting that the GH-IGF-I system is an important contributor to insulin resistance during puberty in boys (Moran et al. 2002).

Androgens

In healthy adult males, suppression of testosterone secretion by a GnRH agonist and concomitant administration of different doses of testosterone leading to physiological testosterone concentrations (Singh et al. 2002) or administration of testosterone with supraphysiological doses (Hobbs et al. 1996) did not affect insulin sensitivity. Moreover, low concentrations of bioavailable testosterone failed to independently predict insulin resistance in normogonadal men (Abate et al. 2002). Existing data suggest that testosterone does not independently regulate insulin sensitivity in adult males. However, testosterone administration has been shown to improve insulin resistance in abdominally obese men simultaneously with a decrease of visceral fat mass (Mårin et al. 1992). The relationship between androgens and glucose-insulin homeostasis in men may, however, be explained to a large extent by concomitant alterations in fat tissue (Tchernof et al. 1995). Since plasma concentrations of free

testosterone are associated with total body fat and truncal and peripheral fat (Abate et al. 2002), testosterone may have a role in the regulation of insulin sensitivity indirectly through its effect on fat tissue in adult males.

Treatment of boys with delayed puberty with testosterone or a nonaromatizable androgen, DHT, did not change insulin sensitivity, suggesting that pubertal insulin resistance is not attributable to androgens (Arslanian et al. 1997; Saad et al. 2001). This conclusion is further supported by the findings of a longitudinal study in which changes in insulin sensitivity in boys during puberty were not associated with changes in testosterone concentrations (Goran et al. 2001). However, in a cross-sectional study, insulin sensitivity was predicted from testosterone concentration (Cook et al. 1993). Although further studies are needed to confirm whether endogenous androgens contribute to the development of puberty-associated insulin resistance in boys, existing evidence suggests that androgens do not have an essential role in the regulation of insulin sensitivity in boys during puberty.

Estrogens

Ethinyl estradiol administration to male-to-female transsexuals decreased insulin sensitivity (Polderman et al. 1994). By contrast, in a man with suppressed estrogen synthesis due to a mutation in the P450 aromatase gene, estrogen treatment decreased previously increased insulin concentration, suggesting an improvement of insulin sensitivity during estrogen administration (Morishima et al. 1995; Bilezikian et al. 1998). The effect of estrogens on insulin sensitivity may be dose-dependent. The available data suggest that physiological concentrations of estrogens may be a prerequisite for normal insulin metabolism.

Data concerning the role of estrogens in the regulation of insulin sensitivity in boys during puberty are very scarce. In a longitudinal study, changes in insulin sensitivity in boys during progression of puberty were not associated with changes in estradiol concentrations (Goran et al. 2001). However, since GH secretion is stimulated by estrogens (Metzger et al. 1994), estrogens may participate in the regulation of insulin sensitivity during puberty indirectly through the GH-IGF-I system.

Regulation of hypothalamic-pituitary-testicular axis

The reproductive system is maintained by the episodic secretion of GnRH from the hypothalamus. GnRH, via hypophyseal-portal blood, induces the release of LH and FSH from the pituitary gland. These stimulate the secretion of gonadal hormones which,

in turn, feed back to the hypothalamus and the pituitary gland, thus participating in the regulation of GnRH and gonadotropin secretion.

Androgens

Patients with complete androgen insensitivity syndrome have increased concentrations of LH, despite normal or high concentrations of estrogens and androgens, but usually have normal concentrations of FSH (Judd et al. 1972; Faiman et al. 1974; LaCroix et al. 1979). These findings indicate that endogenous androgens inhibit LH secretion in adult males, while their role in the control of FSH secretion may be less important. Testosterone infusion decreased gonadotropin concentrations and LH pulse amplitude in men with idiopathic hypogonadotropic hypogonadism whose pituitary-gonadal function had been normalized with long-term pulsatile GnRH replacement, demonstrating that negative feedback of testosterone occurs at the pituitary (Finkelstein et al. 1991). Testosterone administration decreased gonadotropin concentrations to a greater extent in normal men than in those with idiopathic hypogonadotropic hypogonadism and decreased LH pulse frequency in normal men, indicating that the hypothalamus is another site of negative feedback action for testosterone (Finkelstein et al. 1991). However, effects of testosterone can be androgen- or estrogen-mediated. An infusion of a nonaromatizable androgen, DHT, at the blood production rate of testosterone decreased LH concentrations and LH pulse frequency but did not change LH pulse amplitude, which suggests that androgen-mediated negative feedback occurs primarily at the hypothalamus (Veldhuis et al. 1984). Actions of exogenously administered steroids may, however, differ from the effects of endogenous steroids. Inhibition of endogenous androgen-mediated effects by androgen receptor antagonist flutamide increased LH concentration, but exogenous GnRH-induced LH release did not change, suggesting that negative feedback of endogenous androgens also occurs primarily at the site of the hypothalamus (Urban et al. 1988; Veldhuis et al. 1992). Another androgen receptor blocker, Anandron®, increased basal and exogenous LH-releasing hormone-stimulated LH concentrations and LH pulse frequency and amplitude (Gooren et al. 1987). These findings also support endogenous androgens inhibiting gonadotropin secretion at the site of the hypothalamus, but do not exclude the possibility of negative feedback at the pituitary in adult males.

In late pubertal boys, flutamide administration increased LH concentrations but did not change FSH concentrations, probably due to an increase in estradiol concentrations (Kerrigan et al. 1994). Endogenous androgen-mediated inhibition of LH secretion appears to occur primarily at the site of the hypothalamus in late pubertal boys since after inhibition of endogenous androgen action, LH concentrations and pulse frequency increased, but exogenous GnRH-induced LH release remained unchanged

(Kerrigan et al. 1994). Endogenous androgens seem to regulate gonadotropin secretion in males already during the early stages of puberty, as an antiandrogen, spironolactone, increased both LH and FSH concentrations in a group composed of pre-, early, and midpubertal boys (Santen et al. 1976). Moreover, nonaromatizable androgens or anabolic steroids (oxandrolone, DHT, and fluoxymesterone) decreased gonadotropin concentrations in pre- and early pubertal boys (Hopwood et al. 1979; Keenan et al. 1993; Malhotra et al. 1993).

In conclusion, endogenous androgens appear to inhibit gonadotropin secretion from the early stages of puberty onwards. During late puberty endogenous androgen-mediated negative feedback occurs primarily at the site of the hypothalamus, and in adult males, also at the hypothalamus, but possibly at the pituitary as well.

Estrogens

The essential role of endogenous estrogens in the regulation of gonadotropin secretion in adult males was demonstrated with the three different case reports of impaired estrogen action, referred to above (Smith et al. 1994; Morishima et al. 1995; Carani et al. 1997; Bilezikian et al. 1998). In these reports, gonadotropin concentrations were elevated and decreased with estrogen therapy in the men with aromatase deficiency (Smith et al. 1994; Morishima et al. 1995; Carani et al. 1997; Bilezikian et al. 1998). Suppression of estradiol concentrations by the P450 aromatase inhibitor, anastrozole, increased LH and FSH concentrations in normal men and in those with idiopathic hypogonadotropic hypogonadism whose pituitary-gonadal system had been normalized by long-term pulsatile GnRH therapy (Hayes et al. 2000). Despite similar changes in sex steroid concentrations, the increase was greater in the normal men (Hayes et al. 2000). This study proved that in adult males gonadotropin secretion is regulated by endogenous estrogens at both the pituitary and the hypothalamus.

The role of low concentrations of endogenous estrogens in the regulation of gonadotropin secretion in boys during puberty has been unclear. In early and midpubertal boys, the hypothalamic-pituitary axis is responsive to estrogens since estradiol infusion decreases LH concentration (Kletter et al. 1997). When estradiol concentrations were increased in early and midpubertal boys to the adult male range by estradiol infusion, LH pulse frequency decreased, but neither LH pulse amplitude nor the exogenous GnRH-induced LH response changed, indicating that supraphysiological concentrations of circulating estrogens inhibit LH secretion at the site of the hypothalamus (Kletter et al. 1997).

AIMS OF THE STUDY

The objectives of the study were:

1. To investigate prospectively whether combining the P450 aromatase inhibitor, letrozole, which suppresses estrogen synthesis, with testosterone treatment in boys with CDP would delay bone maturation and increase adult height (I).

2. To evaluate the role of sex steroids in boys during puberty in the regulation of

- BMD and bone turnover (IV).
- serum lipid concentrations (III).
- insulin sensitivity (III).
- gonadotropin secretion (II).

PATIENTS AND METHODS

ETHICAL CONSIDERATIONS

The protocol was approved by the Ethics Committee of the Hospital for Children and Adolescents and the National Agency for Medicines. Informed written consent was obtained from the patients and their guardians.

PATIENTS AND STUDY PROTOCOL

The boys were referred to the Hospital for Children and Adolescents, University of Helsinki, for evaluation of delayed puberty and/or short stature. A total of 38 boys were seen at the outpatient clinic; three of these boys refused to enter the study and two were excluded because they had already reached midpuberty (Figure 3). Thus, 33 boys were recruited in all (Table 1). The diagnosis of constitutional delay of puberty was defined as a Tanner genital or pubic hair stage (P) observed at an older age than the mean +2 SD for healthy Finnish boys (Ojajärvi 1982) or a testis volume of less than 4 ml after 13.5 years of age. At entry, none of the boys had had any pubertal increase in growth velocity. The boys whose target height would have been more than +1 SD would have been excluded from the study, but none of the boys fulfilled this criterion. Neither medical history, clinical examination, nor routine laboratory tests revealed any signs of chronic illnesses which could account for the delayed puberty. Twenty-five (76%) of the boys had a family history of delayed puberty. None had received any previous sex hormone treatment. Two boys were receiving inhaled corticosteroid treatment for asthma.

Ten boys with a mean age of 15.0 ± 0.2 years (range, 14.4 to 16.8) decided to wait for spontaneous progression of puberty without medical intervention, and thus, composed the untreated group (Figure 3). Twenty-three boys with a mean age of 15.1 ± 0.2 years (range, 13.5 to 16.1) desired medical intervention and were randomly assigned to receive one of two treatments (Figure 3). The boys in the testosterone-plus-placebo-treated group (12 boys) received testosterone enanthate (Testoviron-Depot-250, Schering, Berlin, Germany) six times at a dose of 1 mg/kg intramuscularly every four weeks, and placebo orally once a day for 12 months (Figure 4). The testosterone-plus-letrozole-treated group (11 boys) received testosterone enanthate (as above) as well as a specific and potent fourth-generation aromatase inhibitor, letrozole (Femar, Novartis AG, Stein, Switzerland, commercially purchased from a hospital pharmacy), 2.5 mg

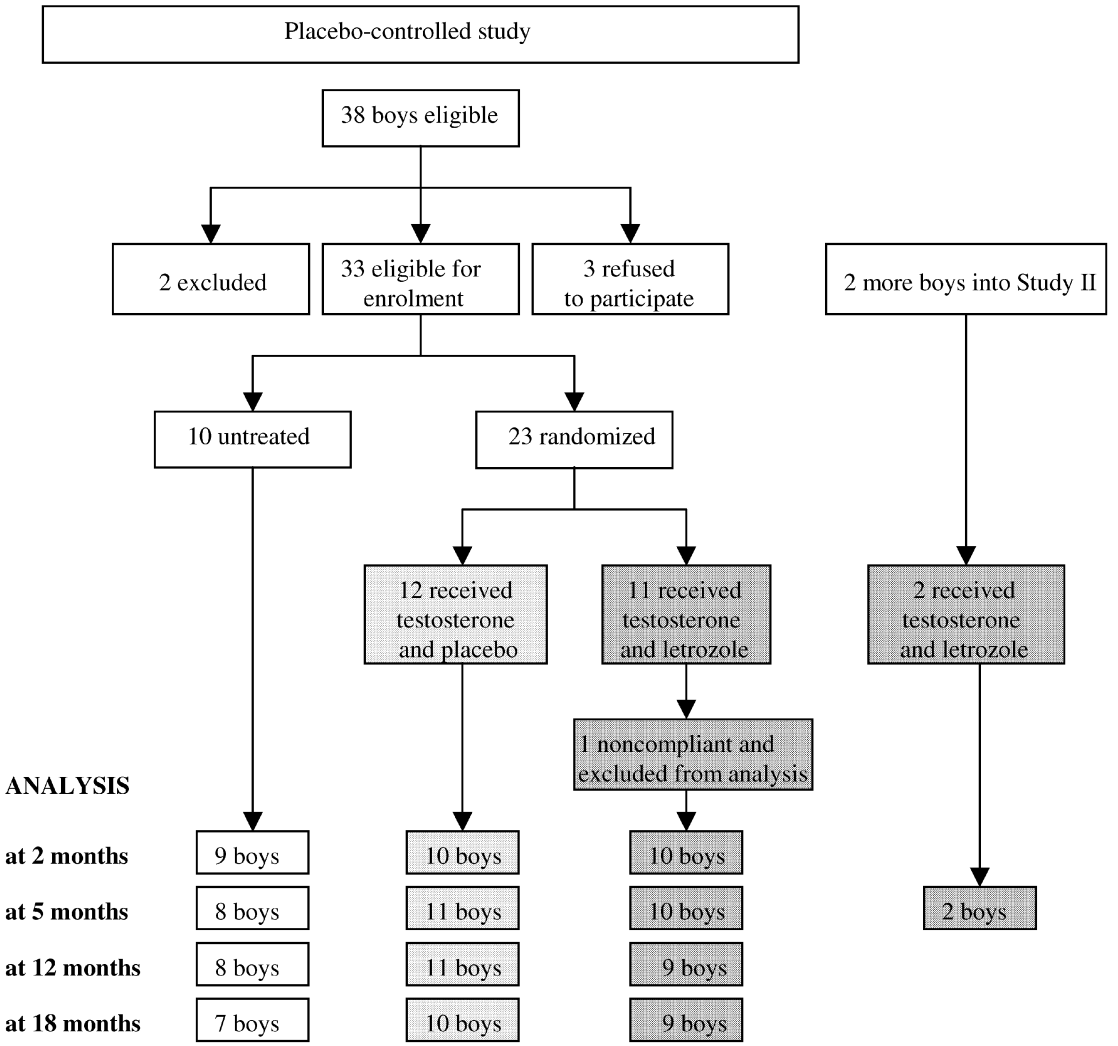

Figure 3. Trial profile.

Table 1. Clinical characteristics of the boys at the start of follow-up.

	No treatment	Testosterone + placebo	Testosterone + letrozole
Chronological age (years)	15.0 ± 0.2	15.0 ± 0.2	15.2 ± 0.2
Bone age (years) [a]	12.7 ± 0.3	12.6 ± 0.4	13.1 ± 0.2
Height (cm)	154.3 ± 1.4	151.9 ± 2.4	155.3 ± 2.1
Pubertal stage [b]	G2(2-3) P2(1-2)	G2(2-3) P1(1-2)	G2(2-3) P1(1-2)
Testis volume (ml) [c]	5.9 ± 0.9	6.9 ± 1.2	5.5 ± 0.6
Predicted adult height (cm) [d]	178.3 ± 1.4	174.9 ± 2.4	176.5 ± 1.7

Mean ± SEM or median (range). [a] By the method of Greulich and Pyle (1959). [b] According to Tanner (1962). [c] From the formula length x width^2 x 0.52 (Hansen et al. 1952). [d] By the Bayley-Pinneau (1952) method.

orally once a day for 12 months (Figure 4). The project was conducted as a randomized, double-blind, placebo-controlled study between the treated groups (I, III, IV).

The subjects were examined at the start and at 2 months (approximately 7 days after the 3rd testosterone injection), 5 months (approximately 7 days after the 6th testosterone injection), 12 months, and 18 months (Figure 4). Nine boys in the untreated group, 10 in the testosterone-plus-placebo-treated group, and 11 in the testosterone-plus-letrozole-treated group completed the 2-month follow-up; 8, 11, and 11 boys, respectively, completed the 5-month follow-up; 8, 11, and 10, respectively, the 12-month follow-up; and 7, 10, and 10, respectively, the 18-month follow-up (Figure 3). One boy in the testosterone-plus-letrozole-treated group was considered noncompliant and his results were excluded from analyses (Figure 3). Since nocturnal gonadotropin pulses had been determined in 5 testosterone-plus-placebo-treated boys and only 3 testosterone-plus-letrozole-treated boys during the randomized, placebo-controlled study, 2 boys were subsequently treated with testosterone and letrozole, and were included in Study II (Figure 3). Both of these boys completed the 5-month follow-up (Figure 3).

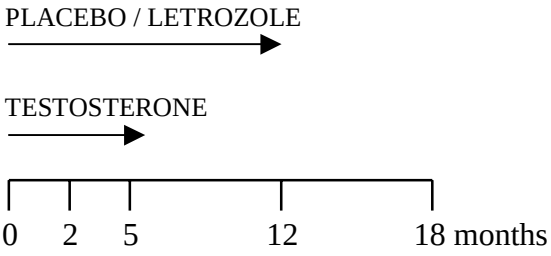

Figure 4. Treatment regimens and time points of follow-up.

LETROZOLE, A P450 AROMATASE INHIBITOR

Letrozole (CGS 20267) is a novel, specific, and potent fourth-generation nonsteroidal aromatase inhibitor which inhibits the conversion of testosterone to 17β-estradiol and of Δ^4androstenedione to estrone. It is mostly metabolized in the liver, and letrozole and its metabolites are excreted mainly via the kidneys. The plasma terminal elimination half-life is approximately 2 days. Steady-state plasma concentrations are reached within 2 to 6 weeks with a dose of 2.5 mg once a day. The current official indication for letrozole is breast cancer. Letrozole has been shown to be well tolerated, and it has no other pharmacological effects *in vivo* (Iveson et al. 1993; Trunet et al. 1993; Lipton et al. 1995; Ingle et al. 1997; Dombernowsky et al. 1998; Gershanovich et al. 1998). Metabolic and hormonal effects of another fourth-generation aromatase inhibitor, anastrozole, in late pubertal boys and adult males (aged 14-22 years) have been studied previously (Mauras et al. 2000).

METHODS

Auxological measurements and staging in puberty (I)

Heights were measured on a Harpenden stadiometer with 0.1 cm precision. Pubertal stages were assessed according to Tanner (1962). Testis volumes were calculated from the formula length x width^2 x 0.52 (Hansen et al. 1952) and were presented as means of the two testes.

Bone age (I)

Bone ages were determined blindly by the method of Greulich and Pyle (1959). Bone age x-ray films of each time point were first ranked in successive order according to maturation, after which the bone age for each film was determined.

Adult height prediction (I)

Adult height predictions were calculated using the Bayley-Pinneau (1952) method; the table for boys with average skeletal maturity was used, as the bone ages in most of the boys exceeded the range of bone ages reported for boys with retarded skeletal maturity. The Bayley-Pinneau method employs tables giving the percentages of final height

acquired at each bone age, as estimated by the method of Greulich and Pyle. This method has been demonstrated to give accurate adult height predictions for Finnish boys with CDP (Lenko 1979).

Body composition (III, IV)

Weight was measured with underwear on. The body mass index (BMI) was calculated from the following formula: weight (kg) / height^2 (m^2). Fat-free mass (FFM), fat mass (FM), and percentage of FM was determined by bioelectrical impedance analysis. FFM was determined with an equation developed by Houtkooper et al. (1992). FM was determined by subtracting FFM from weight. The percentage of body fat was also estimated from the sum of six skinfolds (Anyan 1978) in most of the boys from 2 months onwards. These percentages of body fat and the percentages of body fat measured by the bioelectrical impedance analyses correlated at each time point, validating the measurements obtained by bioelectrical impedance analysis. The changes in FFM and FM could be determined within 5 months in 9 boys treated with testosterone and placebo and in 10 boys treated with testosterone and letrozole, but within 12 months only in 6 and 9 boys, and within 18 months only in 6 and 7 boys, respectively.

Bone mineral density measurement (I, IV)

The bone mineral content (BMC) of the first through fourth lumbar spines and the femoral neck were determined by dual-energy x-ray absorptiometry (Hologic QDR-1000, Hologic Inc., Waltham, MA, USA) at the start and at 5 months, 12 months, and 18 months. The BMD was calculated by dividing the quantity of bone mineral within the scan area (BMC) by the projected area within the region of interest (area). According to the manufacturer, the coefficient of variation of BMD in the lumbar spine is 0.6% and in the femoral neck 1.5% in a normal population. The areal BMD obtained by this method can be confounded by changes in bone thickness. To minimize the contributions of bone dimensions, bone mineral apparent density (BMAD), an estimate of volumetric BMD, was also determined. Lumbar spine BMAD was calculated using the formula BMC ÷ (area)$^{1.5}$, and femoral neck BMAD using the formula BMC ÷ ((area)^2 ÷ 1.6), corresponding to the length of the scanned area (1.6 cm) (Katzman et al. 1991).

Laboratory methods

All of the venous blood samples were drawn between 07:30 and 10:15, typically after a 12-hour fast, but after at least a 10-hour fast. Serum concentrations of bone turnover markers, insulin, and inhibin B were determined at the start and at 5 months, 12 months, and 18 months; all other parameters were measured at every visit.

17β-estradiol (I-IV)

Serum 17β-estradiol concentrations were determined by a modified radio-immunoassay (RIA) using coated tube technology (Spectria estradiol, Orion Diagnostica, Espoo, Finland) after diethyl ether extraction (700 µl serum plus 5 ml diethyl ether) (Norjavaara et al. 1996). The detection limit of the assay was 6 pmol/l. The interassay coefficient of variation (CV) was 27% for concentrations of 8-15 pmol/l, and 17% for concentrations of 15-30 pmol/l. The intra-assay CV ranged from 12% to 16% for concentrations of 8-50 pmol/l.

Testosterone and DHT (I-IV)

Serum testosterone and DHT concentrations were measured by RIA after separation of steroid fractions on a Lipidex-5000 microcolumn (Packard-Becker, B.V. Chemical Operations, Groningen, The Netherlands) (Apter et al. 1976). The inter- and intra-assay CVs for testosterone were 15% at a concentration of 15.6 nmol/l. For DHT, the interassay CV was 17% and the intra-assay CV 14% at a concentration of 2.5 nmol/l.

IGF-I and IGFBP-3 (I, III, IV)

Serum IGF-I and IGFBP-3 concentrations, surrogate markers of GH secretion, have been shown to reflect GH secretion in healthy children (Blum et al. 1993). The concentrations of IGF-I and IGFBP-3 were determined by RIAs (DiaSorin, Stillwater, MN, USA, and Nichols Institute Diagnostics, San Juan Capistrano, CA, USA, respectively). For IGF-I, the interassay CV was 15.2% at a concentration of 32.7 nmol/l and the intra-assay CV 9.1% at a concentration of 24.8 nmol/l. The interassay CV for IGFBP-3 was 12% and the intra-assay CV 5.6% at a concentration of 4.1 mg/l.

Bone turnover markers (IV)

Measuring serum concentrations of bone turnover markers offers an indirect way to study various aspects of bone metabolism. Type I collagen degradation and bone resorption are mirrored by a degradation product, cross-linked carboxyterminal telopeptide of type I collagen, which was measured by two different methods (CTx and

ICTP) (Eriksen et al. 1993; Risteli et al. 1999). The rate of type I collagen synthesis, which accompanies bone formation, is reflected by the serum concentration of a synthesis by-product, carboxyterminal propeptide of type I procollagen (PICP) (Eriksen et al. 1993; Risteli et al. 1999). Bone formation was also estimated by measuring serum concentrations of osteocalcin (OC) (Brown et al. 1984) and alkaline phosphatase (ALP), the bone isoform of which constitutes about 75-90% of total serum ALP activity in children over the age of 4 years (Schönau et al. 1997).

Serum CTx concentrations were measured by Serum CrossLaps ELISA (Nordic Bioscience Diagnostics, Herlev, Denmark). The interassay CV for CTx was 7.1% at a concentration of 4.2 nmol/l and the intra-assay CV 5.5% at a concentration of 5.6 nmol/l. Serum ICTP and PICP concentrations were determined by RIA (Orion Diagnostica, Espoo, Finland). The interassay CV for ICTP was 8.0% at a concentration of 21.2 μg/l and the intra-assay CV 4.1% for concentrations of 10-100 μg/l. The interassay CV for PICP was 5.2% at a concentration of 191 μg/l and the intra-assay CV 3.0% for concentrations of 50-400 μg/l. The serum OC concentrations were measured by an immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). The interassay CV for OC was 4.4% and the intra-assay CV 3.6% at a concentration of 73 μg/l. Serum ALP concentrations were determined using a Hitachi 917 Modular autoanalyzator. The interassay CV for ALP was 9.3% at a concentration of 729 U/l.

Lipids (III)

Concentrations of serum total cholesterol, HDL-cholesterol, and triglycerides were determined by enzymatic colorimetric tests (Roche Diagnostics GmbH, Mannheim, Germany). The interassay CV for total cholesterol was 1.7% at a concentration of 5.5 mmol/l and the intra-assay CV 0.8% at a concentration of 6.0 mmol/l. The inter- and intra-assay CVs for HDL-cholesterol were 2.6% and 1.3%, respectively, at a concentration of 0.6 mmol/l, and for triglycerides 1.8% (2.5 mmol/l) and 1.5% (2.3 mmol/l), respectively. Serum LDL-cholesterol concentrations were calculated using the equation of Friedewald et al. (1972).

Insulin (III)

Serum insulin concentrations were determined by RIA (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). The intra-assay CV and total CV for insulin were 8.0% at a concentration of 20.4 mU/l.

Gonadotropins (I, II)

Serum LH and FSH concentrations were measured by ultra-sensitive immunofluorometric assay (Wallac, Turku, Finland) (Dunkel et al. 1990b). The sensitivity of the assay for LH and FSH was 0.05 IU/l, as defined by the mean +2 SD of 20 (LH) or 96 (FSH) replicates of a zero sample. The interassay CV for LH ranged from 3.2% to 4.6% at concentrations of 2.0-54.0 IU/l, and the interassay CV for FSH from 2.6% to 3.3% at concentrations of 6.6-35.1 IU/l. The intra-assay CV was calculated by measuring 12 or 36 replicates at 3 different concentrations of LH and FSH. The intra-assay CV for LH was 4.1% at a concentration of 0.35 IU/l (n=36), 2.0% at a concentration of 3.0 IU/l (n=36), and 1.6% at a concentration of 8.7 IU/l (n=12). The intra-assay CV for FSH was 4.4% (0.25 IU/l; n=36), 1.8% (2.9 IU/l; n=36), and 1.6% (6.7 IU/l; n=12). These results were used for calculating the assay SD coefficients for the pulse analysis program. LH concentrations of less than 0.1 IU/l were treated as 0.1 IU/l.

Inhibin B (I, II)

Serum inhibin B concentrations were determined by enzyme-linked immunosorbent assay (Serotec, Oxford, UK). The interassay CV was less than 8% at concentrations of 115 ng/l and 282 ng/l, and the intra-assay CV was less than 5% at concentrations of 115 ng/l and 272 ng/l.

GnRH test (II)

At the start, at 5 months, and at 12 months, GnRH (Relefact®, 3.5 μg/kg, maximum 100 μg; Hoechst Marion Roussel, Deutschland GmbH, Frankfurt, Germany) was administered intravenously, and LH concentrations were measured from samples obtained at 0 (before), 20, 30, and 60 minutes and FSH concentrations at 0 (before), 30, 60, and 90 minutes after administration of GnRH in all boys. The GnRH tests were started between 07:30 and 10:15. The GnRH-induced gonadotropin response was defined as the difference between the basal and the GnRH-induced peak gonadotropin concentrations.

Gonadotropin pulse analysis (II)

Nocturnal gonadotropin pulses were studied at the start and at 5 months in 5 boys treated with testosterone and placebo and in 5 boys treated with testosterone and

letrozole. For determination of nocturnal gonadotropin pulses, an indwelling intravenous cannula was inserted approximately one hour before the beginning of sampling. Sleep was monitored visually by trained nursing personnel. The serum LH and FSH concentrations were determined every 15 minutes. One boy fell asleep at 03:10, at the time point before the treatment, the other boys fell asleep before midnight.

The serum LH and FSH concentrations from each individual at a given time point were analyzed in the same assay. LH and FSH pulsations were analyzed by a computerized pulse analysis program, Munro (Zaristow Software, East Lothian, Scotland). The program identifies secretory peaks by height and duration from a smoothed baseline, using the assay SD as a scale factor. Munro is an adaptation of the Pulsar Program developed by Merriam and Wachter (1982). The only essential difference is in the calculation of the baseline; in the Munro program, the baseline is generated by linear interpolation between the nadirs, followed by smoothing, using a moving average. The remaining stages of the Munro algorithm are identical with those of the Pulsar program. As the baseline in the Munro program is calculated from the nadirs rather than from the moving average of the data, this program can process data containing pulses of variable widths and amplitudes. This is considered essential in the analysis of FSH pulses, which are wider than LH pulses. The cut-off parameters G1-5 of the Munro program for LH were set at 3.98, 2.4, 1.68, 1.24, and 0.93, and those for FSH were set at 10.0, 2.4, 1.68, 1.24, and 0.93 times the intra-assay SD as criteria for accepting peaks 1, 2, 3, 4, and 5 points wide, respectively. The smoothing time, a window used to calculate the moving average, was set at 135 minutes, i.e., 9 data points wide for both LH and FSH. With these settings, the program did not detect any peaks when 33 consecutive samples from plasma pools with LH concentrations of approximately 0.35 IU/l and 3.0 IU/l and with FSH concentrations of approximately 0.25 IU/l and 2.9 IU/l were assayed. Thus, use of a special program for minimizing false-positive error in pulse detection (Veldhuis et al. 1985) was not deemed necessary. Missing values comprised approximately 0.6% of total samples and were left blank. The interpulse interval was defined as the time interval between consecutive peaks.

Statistical analysis

Values are expressed as means ± standard error of the mean (SEM) unless otherwise reported. Analyses were conducted with the SPSS statistical software for Windows, Release 8.0.2 (I, II, III) or Release 10.0.7 (IV) (SPSS, Inc., Chicago, IL, USA). One-way analyses of variance, Student's unpaired t-test, Kruskal-Wallis nonparametric analyses of variance, or Mann-Whitney U-test were used as appropriate in studying the

differences between groups at the start or at the time point of 18 months. Student's paired t-test or the Wilcoxon matched pairs signed rank test were used for analyses of the changes within groups during the follow-up. For analysis of serial measurements, the summary measures, the differences from the start, were calculated for each subject, and these values were treated as raw data for the appropriate statistical analysis; Student's unpaired t-test or Mann-Whitney U-test were used as appropriate. Pearson's correlation coefficient was used to investigate the relationship between growth velocity and hormone concentrations (I). Pearson's or Spearman's correlations were used to assess the relationships between changes in insulin concentrations and changes in BMIs, concentrations of 17β-estradiol, testosterone, IGF-I, and IGFBP-3, and the relationships between changes in concentrations of HDL-cholesterol and changes in BMIs, concentrations of 17β-estradiol, testosterone, IGF-I, and IGFBP-3 (III). To assess the relationship of BMD with hormonal factors and BMI, the changes in lumbar spine and femoral neck BMAD between 0 and 12 months were compared with the means of serum concentrations of 17β-estradiol, testosterone, DHT, IGF-I, IGFBP-3, and BMI at 2, 5, and 12 months by Spearman's correlation (IV). All statistical tests were two-sided. A p-value of less than 0.05 was considered statistically significant.

RESULTS

SAFETY OF LETROZOLE

For detecting possible side-effects of letrozole, the concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, and transaminases, the leukocyte count, and the BMD were determined during the follow-up. No changes sufficient to indicate discontinuation of treatment were observed in any of these parameters. Letrozole was well tolerated; no side-effects were observed.

17β-ESTRADIOL REFLECTING TREATMENT EFFECT (I-IV)

Letrozole inhibited estrogen synthesis effectively (Figure 5). During treatment with testosterone and placebo the 17β-estradiol concentration increased (16.4 ± 2.9 pmol/l at start, 37.9 ± 15.1 pmol/l at 5 months; probability (p) =0.02), and an increase was also observed in the untreated group during follow-up. In contrast, during treatment with testosterone and letrozole the concentration remained at the pretreatment level (12.8 ± 3.0 pmol/l at start, 10.2 ± 1.3 pmol/l at 5 months, p=0.3). After discontinuation of letrozole treatment, the 17β-estradiol concentration also increased in the testosterone-plus-letrozole-treated group, and at 18 months, i.e., 6 months after discontinuation of all treatments, the 17β-estradiol concentrations in all groups were similar.

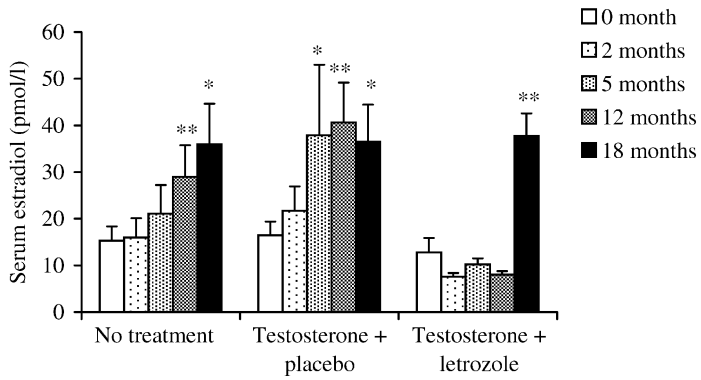

Figure 5. Serum 17β-estradiol concentration (mean + SEM). Asterisks denote significant changes from the start within each group: *, p<0.05; **, p<0.01.

TESTOSTERONE AND DHT (I-IV)

The testosterone concentrations increased in all three groups, but during treatment with testosterone and letrozole the increase was more than 5-fold higher than during treatment with testosterone and placebo (Figure 6). In the testosterone-plus-placebo-treated group, the testosterone concentration was 11.9 ± 2.9 nmol/l at the start and 18.4 ± 3.1 nmol/l at 5 months (p=0.02), and in the testosterone-plus-letrozole-treated group, 9.5 ± 3.5 nmol/l at the start and 65.5 ± 13.1 nmol/l at 5 months (p=0.001). In the testosterone-plus-letrozole-treated group, the high concentration was sustained until discontinuation of letrozole treatment, after which the concentration decreased to a level comparable with those of the other groups. DHT concentrations had similar patterns of changes to testosterone concentrations, although the magnitude of the changes was smaller (IV).

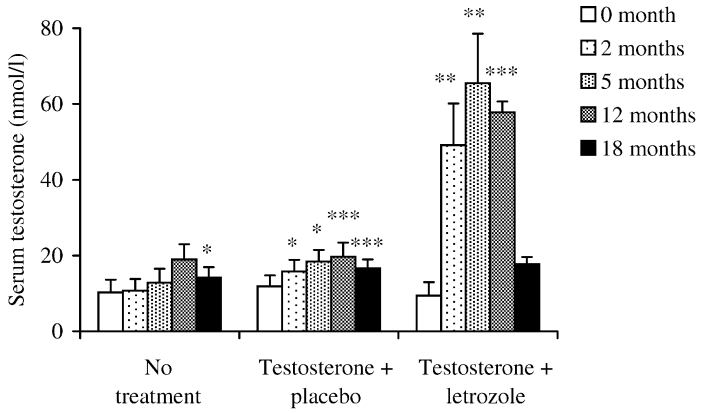

Figure 6. Serum testosterone concentration (mean + SEM). Asterisks denote significant changes from the start within each group: *, p<0.05; **, p<0.01; ***, p<0.001.

IGF-I AND IGFBP-3 (I, III, IV)

The changes in IGF-I and IGFBP-3 concentrations were different in the two treated groups (Table 2). During treatment with testosterone and placebo both concentrations increased immediately after the start of treatment, whereas during treatment with testosterone and letrozole, the concentrations remained at the pretreatment level. Six months after discontinuation of all treatments, i.e., at 18 months, IGF-I and IGFBP-3 concentrations were similar in both treated groups.

Table 2. Serum IGF-I and IGFBP-3 concentrations.

	No treatment	Testosterone + placebo	Testosterone + letrozole	p-value [a]
IGF-I (nmol/l)				
0 month	27.4 ± 3.8	28.3 ± 2.7	30.3 ± 3.4	
2 months	28.7 ± 2.9	34.0 ± 2.4[b]	25.6 ± 1.5	0.01
5 months	25.9 ± 2.0	34.5 ± 2.3[b]	25.2 ± 1.6	0.01
12 months	29.3 ± 3.3	34.3 ± 2.9[b]	27.4 ± 1.0	0.06
18 months	27.9 ± 2.6	31.9 ± 2.6	34.1 ± 1.2	0.9
IGFBP-3 (mg/l)				
0 month	3.7 ± 0.2	3.8 ± 0.1	3.7 ± 0.2	
2 months	3.7 ± 0.3	4.1 ± 0.2[b]	3.6 ± 0.2	0.02
5 months	3.8 ± 0.2	4.3 ± 0.2[c]	3.4 ± 0.2	0.0004
12 months	3.9 ± 0.2	4.3 ± 0.1[d]	3.5 ± 0.2	0.008
18 months	4.5 ± 0.2[b]	4.7 ± 0.2[c]	4.4 ± 0.2[c]	0.8

Mean ± SEM. [a] p-value refers to the difference between treatment groups regarding changes in value from the start to the time point indicated by the p-value. [b] $p<0.05$, [c] $p<0.001$, [d] $p<0.01$ for change within each group from the start to the indicated time point.

GROWTH (I)

From the start to 5 months of treatment, the boys treated with testosterone and placebo grew slightly faster than those treated with testosterone and letrozole (9.9 ± 0.5 cm/year vs. 7.3 ± 0.9 cm/year, respectively, p=0.02; Table 3). After 5 months, no statistically significant differences in growth velocity were observed between the two treated groups, although a trend of higher growth velocity was observed in the testosterone-plus-letrozole-treated than in the testosterone-plus-placebo-treated group after discontinuation of treatments (p=0.06; Table 3). No correlation was found between height velocity during the first 5 months and serum IGF-I, IGFBP-3, 17β-estradiol, testosterone, or DHT concentration at 5 months.

BONE AGE (I)

Inhibition of estrogen synthesis by letrozole delayed bone maturation. Within the 18-month follow-up period, bone age increased 1.7 ± 0.3 years in the testosterone-plus-placebo-treated group but only 0.9 ± 0.2 years in the testosterone-plus-letrozole-treated group (significance of the difference between treatment groups, p=0.03; Figure 7). In the untreated group, the respective increment was 1.1 ± 0.3 years (Figure 7).

Table 3. Growth velocity and predicted adult height.

	No treatment	Testosterone + placebo	Testosterone + letrozole	p-value [a]
Growth velocity (cm/year)				
0 – 5 months	7.3 ± 0.8	9.9 ± 0.5	7.3 ± 0.9	0.02
5 – 12 months	6.6 ± 1.0	7.9 ± 0.8	7.1 ± 0.8	0.5
12 – 18 months	6.5 ± 1.3	6.5 ± 0.5	8.3 ± 0.8	0.06
Predicted adult height (cm) [b]				
0 month	178.3 ± 1.4	174.9 ± 2.4	176.5 ± 1.7	
12 months	176.8 ± 1.2	174.4 ± 2.4	178.0 ± 2.1	0.4
18 months	180.3 ± 1.9	175.2 ± 2.1	182.1 ± 2.1[c]	0.04

Mean ± SEM. [a] p-value refers to the difference between treatment groups regarding growth velocity or changes in value from the start to the time point indicated by the p-value (predicted adult height). [b] By the Bayley-Pinneau (1952) method. [c] p=0.004 for change within the group from the start to the indicated time point.

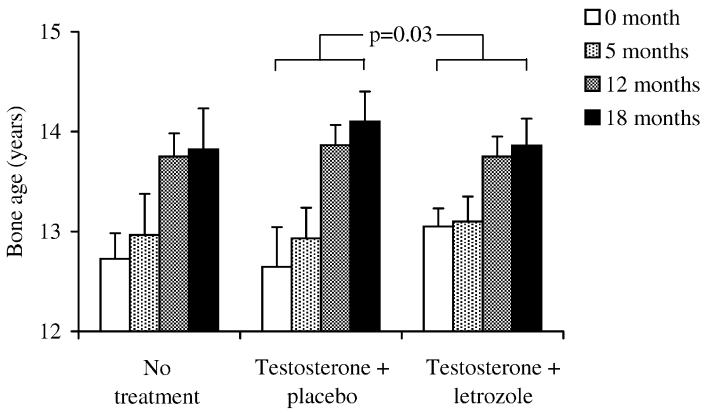

Figure 7. Bone age (mean + SEM). p-value refers to the difference between treatment groups regarding changes in bone age at 18 months.

ADULT HEIGHT PREDICTION (I)

Letrozole treatment increased predicted adult height (Table 3, Figure 8). In the testosterone-plus-placebo-treated group or in the untreated group, the predicted adult height did not change during 18 months of follow-up (Table 3; Figure 8). By contrast, in the testosterone-plus-letrozole-treated group, an increase of 5.1 ± 1.2 cm (p=0.004) in predicted adult height was seen; in one patient, the predicted adult height decreased by 3.5 cm, and the increases in the other boys ranged from 2.5 cm to 8.8 cm. The

difference between the treatment groups regarding the change in predicted adult height was significant (p=0.04).

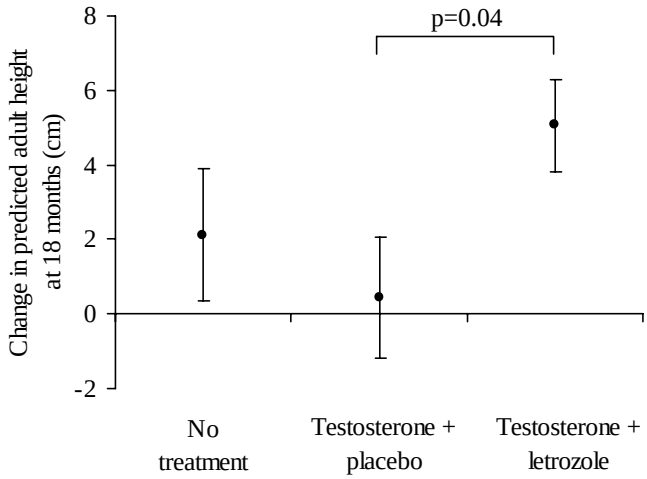

Figure 8. Change in predicted adult height (mean ± SEM) from the start to 18 months.

PROGRESSION OF PUBERTY (I)

Puberty advanced in all groups during the follow-up. The Tanner stages of puberty progressed in a similar fashion in the two treatment groups (I). The increase in testis volume was greater during treatment with testosterone and letrozole than during treatment with testosterone and placebo, which is consistent with the differences in gonadotropin concentrations (Table 4). Gynecomastia was found in 2 boys in the testosterone-plus-placebo-treated group, in 2 boys in the testosterone-plus-letrozole-treated group, and in none of the boys in the untreated group.

BODY COMPOSITION (III, IV)

In the testosterone-plus-placebo-treated group, BMI did not change during the follow-up (III). In the testosterone-plus-letrozole-treated group, BMI was higher during letrozole treatment than at the start, but 6 months after discontinuation of letrozole treatment it did not differ from the pretreatment level (III).

There were no differences between the two treated groups in increases of FFM. The decreases in FM and in the percentage of FM were more profound in the

testosterone-plus-placebo-treated than in the testosterone-plus-letrozole-treated group from the start to 5 months but similar at the other time points (III).

Table 4. Testis volume and serum inhibin B concentration.

	No treatment	Testosterone + placebo	Testosterone + letrozole	p-value [a]
Testis volume (ml) [b]				
0 month	5.9 ± 0.9	6.9 ± 1.2	5.5 ± 0.6	
5 months	8.1 ± 1.4	8.9 ± 1.5	11.5 ± 1.6	0.01
12 months	12.0 ± 1.8	13.4 ± 1.7	16.8 ± 1.2	0.0005
18 months	14.7 ± 2.3	18.6 ± 2.0	19.1 ± 1.1	0.2
Inhibin B (ng/l)				
0 month	153.7 ± 12.1	176.1 ± 12.5	161.2 ± 16.2	
5 months	186.4 ± 18.1	155.5 ± 21.8	200.5 ± 18.8	0.01
12 months	184.6 ± 10.3[c]	186.6 ± 19.0	219.8 ± 15.9[d]	0.1
18 months	180.6 ± 11.4[d]	216.8 ± 19.1	203.1 ± 16.6	0.8

Mean ± SEM. [a] p-value refers to the difference between treatment groups regarding changes in value from the start to the time point indicated by the p-value. [b] From the formula length x width^2 x 0.52 (Hansen et al. 1952). [c] $p<0.01$, [d] $p<0.05$ for change within each group from the start to the indicated time point.

BONE METABOLISM (I, IV)

Bone mineral density (I, IV)

Lumbar spine

In both treatment groups, BMC and bone area increased during the treatments (Figure 9). An increase was also observed in BMD and BMAD in both treated groups during the follow-up, although in the testosterone-plus-letrozole-treated group, the increase in BMAD was statistically significant only at 18 months, i.e. 6 months after discontinuation of letrozole treatment (Figure 9). When the changes from the start in BMC, bone area, BMD, and BMAD were compared between the treated groups, no statistically significant differences were observed at any time point.

To study the association of BMAD with hormonal factors and BMI, the changes in BMAD between 0 and 12 months were compared with the means of 17β-estradiol, testosterone, DHT, IGF-I, IGFBP-3, and BMI at 2, 5, and 12 months in a group including all boys. The changes in lumbar spine BMAD correlated with 17β-estradiol (correlation coefficient (r) =0.5, p=0.009) and IGF-I (r=0.5, p=0.01) concentrations, but no significant associations with other variables were observed.

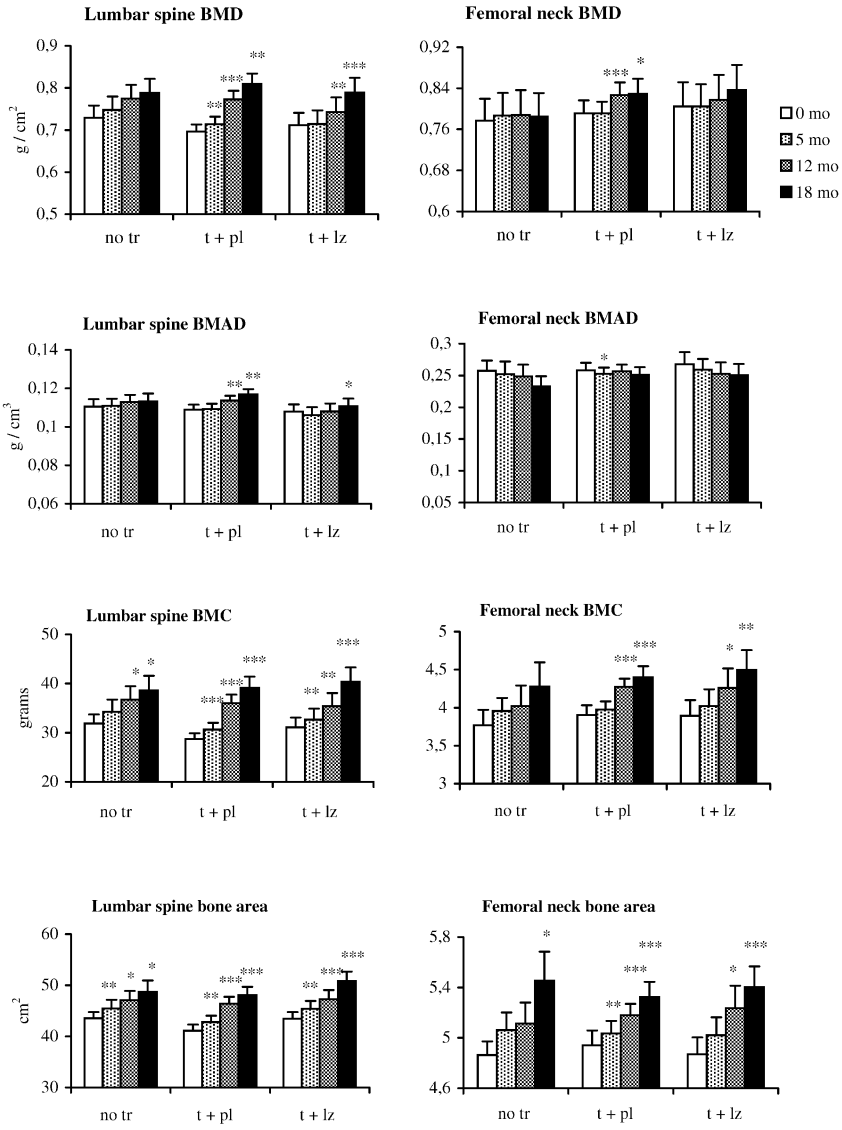

Figure 9. Lumbar spine and femoral neck BMD (areal BMD), BMAD (volumetric BMD), BMC, and bone area (mean + SEM). Boys in the no group did not receive any treatment, boys in the t + pl group received testosterone and placebo, and boys in the t + lz group received testosterone and letrozole. Asterisks denote significant changes from the start within each group: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Femoral neck

BMC and bone area increased during treatment in both treated groups (Figure 9). BMD increased in the testosterone-plus-placebo-treated group, but in the testosterone-plus-letrozole-treated group, no change was observed (Figure 9). BMAD did not change significantly in any of the groups, except for a decrease in the testosterone-plus-placebo-treated group from the start to 5 months (Figure 9). However, a decreasing trend in BMAD in all groups was observed (Figure 9). When the changes from the start in BMC, bone area, BMD, and BMAD were compared between the treated groups, no differences were observed.

The correlations between changes in femoral neck BMAD between 0 and 12 months and the means of 17β-estradiol, testosterone, DHT, IGF-I, IGFBP-3, and BMI at 2, 5, and 12 months were determined in a group including all boys. The only significant association was observed between changes in femoral neck BMAD and IGFBP-3 concentration ($r=0.4$, $p=0.02$).

Bone turnover (IV)

During treatment with testosterone and placebo a simultaneous increase occurred in both bone resorption and formation markers (Figure 10). During treatment with testosterone and letrozole the concentrations of CTx, PICP, and OC remained unchanged, whereas an increase was seen in the concentrations of ICTP and ALP (Figure 10). The untreated group showed a similar pattern of changes; i.e., no change in the concentrations of CTx, PICP, and OC but an increase in ICTP and ALP concentrations.

LIPIDS (III)

The HDL-cholesterol concentration decreased more during the treatment with testosterone and letrozole than during the treatment with testosterone and placebo (Table 5). In the testosterone-plus-placebo-treated group, no significant change in the HDL-cholesterol concentration was observed during the follow-up; the concentration was 1.56 ± 0.09 mmol/l at the start, and the lowest value of 1.40 ± 0.12 mmol/l was observed at 18 months ($p=0.2$; Table 5). In the testosterone-plus-letrozole-treated group, the concentration decreased from 1.62 ± 0.12 mmol/l at the start to the lowest level of 1.20 ± 0.09 mmol/l at 5 months ($p=0.002$), but no subsequent decrease was observed (1.34 ± 0.09 mmol/l at 18 months; Table 5). In the untreated group, the concentration

decreased from 1.77 ± 0.15 mmol/l at the start to the lowest value of 1.43 ± 0.09 mmol/l at 18 months (p=0.047; Table 5). At 18 months, i.e. 6 months after discontinuation of all treatments, the concentrations were similar in all three groups.

To assess the relationship between HDL-cholesterol concentration and BMIs, sex steroid concentrations, and growth parameters, the changes from the start in concentrations of HDL-cholesterol were compared with changes in BMIs, concentrations of 17β-estradiol, testosterone, IGF-I, and IGFBP-3; the data for the boys in the two treatment groups are combined. Changes in HDL-cholesterol concentrations correlated negatively with changes in BMIs (r=-0.6, p=0.01 within 2 months; r=-0.6, p=0.004 within 5 months; r=-0.4, p=0.08 within 12 months), negatively with changes in testosterone concentrations (r=-0.6, p=0.007 within 2 months; r=-0.6, p=0.004 within 5 months; r=-0.4, p=0.09 within 12 months), positively with changes in IGF-I concentrations (r=0.5, p=0.01 within 5 months), and positively with changes in IGFBP-3 concentrations (r=0.6, p=0.005 within 5 months; r=0.4, p=0.07 within 18 months). No association between HDL-cholesterol and 17β-estradiol concentrations was observed.

The concentrations of LDL-cholesterol or triglycerides did not change during the follow-up in any of the three groups (Table 5).

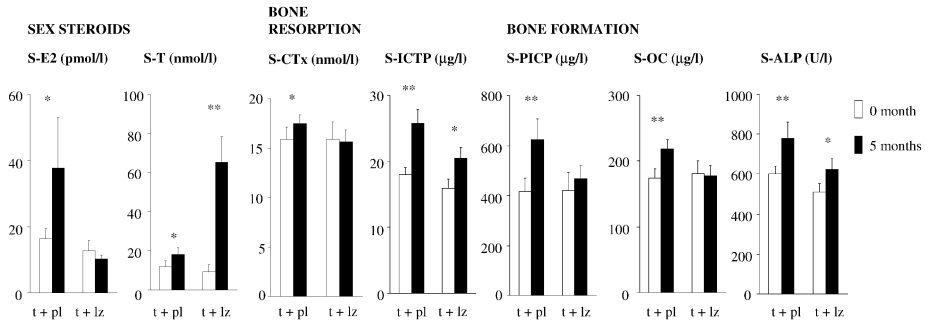

Figure 10. Serum 17β-estradiol, testosterone, CTx, ICTP, PICP, OC, and ALP concentrations (mean + SEM) at the start and at 5 months. Boys in the t + pl group received testosterone and placebo, and boys in the t + lz group received testosterone and letrozole. Asterisks denote significant changes within each group: *, $p<0.05$; **, $p<0.01$.

INSULIN (III)

In the testosterone-plus-placebo-treated group, the fasting insulin concentration remained unchanged (Table 5). By contrast, in the testosterone-plus-letrozole-treated group, the fasting insulin concentration decreased during letrozole treatment (p=0.04, from the start to 12 months), and after discontinuation of letrozole treatment, increased to the pretreatment level (Table 5). The changes in fasting insulin concentration from the start to 12 months in the two treatment groups were different (p=0.02).

To investigate the relationship between fasting insulin concentrations and BMIs, sex steroids, and growth factors, the changes from the start in fasting insulin concentrations were compared with the changes in BMIs, concentrations of 17β-estradiol, testosterone, IGF-I, and IGFBP-3; the data for the boys in the two treatment groups are combined. The changes in serum insulin and IGF-I concentrations from the start to 12 months and 18 months were correlated (r=0.5, p=0.04 for both). No associations between other variables were observed.

Table 5. Serum concentrations of HDL-cholesterol, LDL-cholesterol, triglycerides, and insulin.

	No treatment	Testosterone + placebo	Testosterone + letrozole	p-value [a]
HDL-cholesterol (mmol/l)				
0 month	1.8 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	
5 months	1.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.1[b]	0.005
12 months	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.1[c]	0.06
18 months	1.4 ± 0.1[d]	1.4 ± 0.1	1.3 ± 0.1[c]	0.3
LDL-cholesterol (mmol/l)				
0 month	2.2 ± 0.2	2.4 ± 0.2	2.5 ± 0.2	
5 months	2.7 ± 0.2	2.4 ± 0.2	2.5 ± 0.2	0.5
12 months	2.5 ± 0.2	2.4 ± 0.2	2.6 ± 0.2	0.4
18 months	2.3 ± 0.2	2.4 ± 0.2	2.3 ±0.2	0.9
Triglycerides (mmol/l)				
0 month	0.58 ± 0.06	0.91 ± 0.12	0.89 ± 0.15	
5 months	0.62 ± 0.08	0.86 ± 0.12	0.96 ± 0.14	0.6
12 months	0.70 ± 0.17	0.89 ± 0.15	0.85 ± 0.11	0.6
18 months	0.69 ± 0.11	0.85 ± 0.10	1.13 ± 0.18	0.3
Insulin (mU/l)				
0 month	5.7 ± 0.8	7.1 ± 0.7	9.2 ± 1.3	
5 months	6.2 ± 1.2	6.8 ± 0.7	6.2 ± 0.9	0.2
12 months	6.6 ± 1.4	9.1 ± 1.2	6.5 ± 0.8[d]	0.02
18 months	7.6 ± 1.7	8.8 ± 1.2	9.4 ± 1.3	0.3

Mean ± SEM. [a] p-value refers to the difference between treatment groups regarding changes in value from the start to the time point indicated by the p-value. [b] $p<0.01$, [c] $p<0.02$, [d] $p<0.05$ for change within each group from the start to the indicated time point.

GONADOTROPIN SECRETION (I, II)

Basal and GnRH-induced gonadotropin secretion (II)

The patterns of the changes in gonadotropin concentration during the two treatments were different. In the testosterone-plus-placebo-treated group, after treatment for 5 months, simultaneously with a 55% increase in the testosterone concentration (p=0.02) and a 130% increase in the 17β-estradiol concentration (p=0.02), the basal LH concentration decreased by 68% (p=0.01; Figure 11A) and the basal FSH concentration by 70% (p=0.003; Figure 11B). Despite the decrease in basal gonadotropin concentrations, the GnRH-induced LH and FSH responses remained unchanged (Figure 12).

In the testosterone-plus-letrozole-treated group, after treatment for 5 months, simultaneously with an increase of 606% in testosterone concentration (p=0.0005) and unchanged concentrations of 17β-estradiol (p=0.2), the basal LH concentration increased by 208% (p=0.001; Figure 11A), the basal FSH concentration by 167% (p=0.0005; Figure 11B), and the GnRH-induced LH response by 73% (p=0.0005; Figure 12), but the GnRH-induced FSH response did not change significantly (p=0.08; Figure 12). At 12 months, during treatment with letrozole alone, the basal gonadotropin concentrations and their responses to GnRH were similar to those at 5 months.

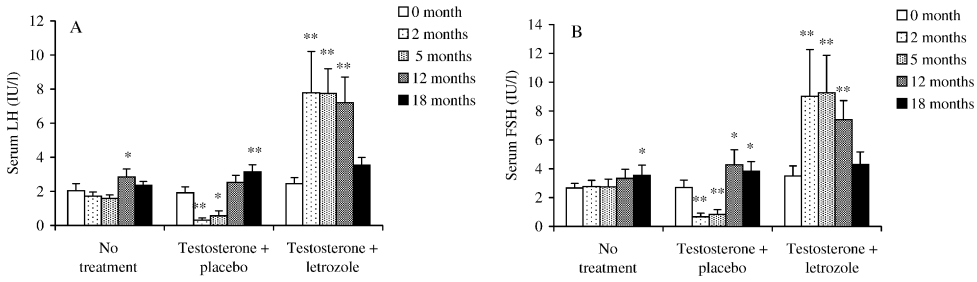

Figure 11. Serum basal LH (A) and FSH (B) concentrations (mean + SEM). Asterisks denote significant changes from the start within each group: *, p<0.05; **, p<0.01.

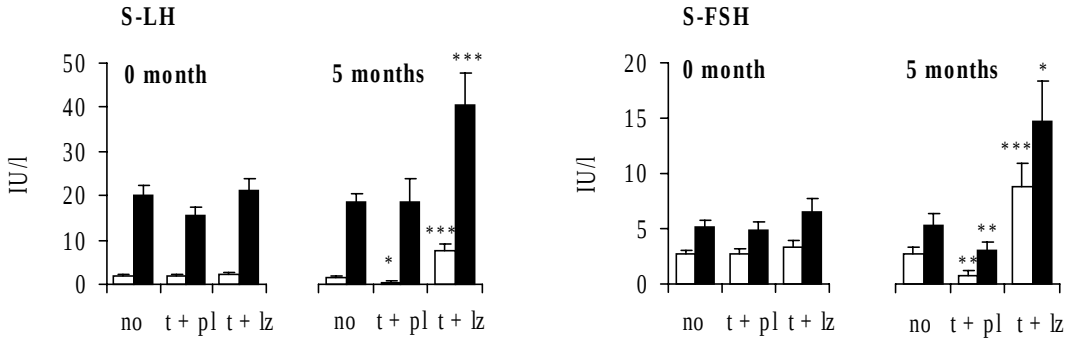

Figure 12. Serum basal (□) and GnRH-induced peak (■) LH and FSH concentrations (mean + SEM) at the start and at 5 months. Boys in the no group did not receive any treatment, boys in the t + pl group received testosterone and placebo, and boys in the t + lz group received testosterone and letrozole. Asterisks denote significant changes from the start within each group: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Spontaneous gonadotropin pulses (II)

The gonadotropin pulses of a representative boy of both treatment groups are shown in Figure 13. Before treatment, nocturnal elevation in gonadotropin concentrations, characteristic of early and midpubertal boys, was seen in all of the boys. During treatment with testosterone and placebo the nocturnal LH concentrations were lower than at the start of treatment in 4 of the 5 boys, and the FSH concentrations in all of the boys. In 3 of the boys in the testosterone-plus-placebo-treated group, the LH concentrations decreased to or below the detection limit, and therefore, no pulses could be determined, but in the other 2 boys, gonadotropin pulses and nocturnal increases in concentrations were observed during treatment. Since pulses could be observed in only 2 of the 5 boys during treatment with testosterone and placebo, the changes in the pulse variables were not calculated.

In the testosterone-plus-letrozole-treated group, the nocturnal LH and FSH concentrations increased in all of the boys during treatment; gonadotropin pulses and the diurnal profile of gonadotropin secretion were also observed. In the testosterone-plus-letrozole-treated group, the mean LH pulse amplitude, calculated from all of the LH pulse amplitudes detected, increased during treatment ($p=0.01$); no significant changes were seen in FSH pulse amplitude, gonadotropin pulse frequencies, or interpulse intervals.

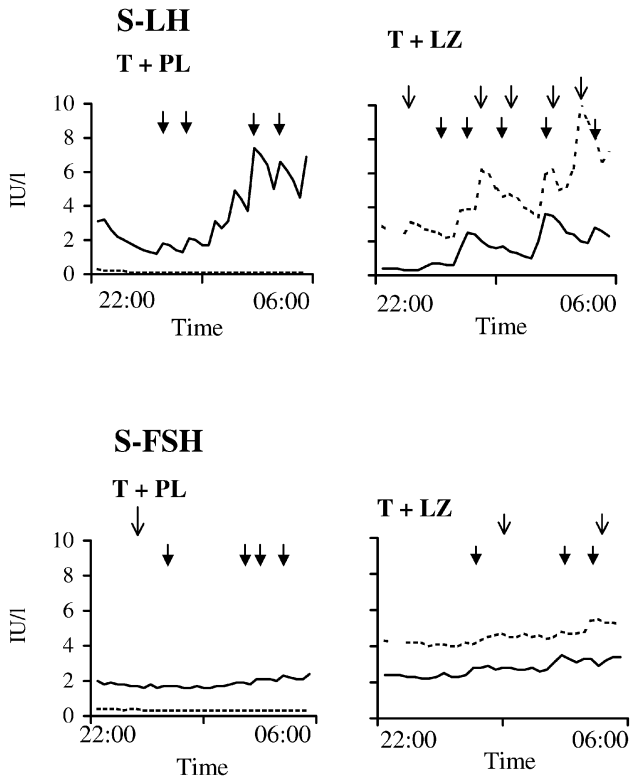

Figure 13. Serum LH (upper panel) and FSH (lower panel) concentrations of a representative boy in both treatment groups. The concentrations are determined every 15 minutes between 22:00 and 06:00 before (solid line) and after (dashed line) 5 months of treatment. Arrowheads denote significant pulses before (closed) and after (open) 5 months of treatment. t + pl, testosterone and placebo; t + lz, testosterone and letrozole.

INHIBIN B (I, II)

In the testosterone-plus-placebo-treated group, the inhibin B concentration did not change significantly (Table 4). In contrast, during the treatment with letrozole, the concentration was higher than at the start, but after discontinuation of the letrozole treatment, it did not differ from the pretreatment concentration (Table 4). The changes in inhibin B concentration from the start to 5 months were different in the two treatment groups (p=0.01), consistent with the difference in increases in testis volume and the divergent pattern of change in gonadotropin concentrations.

DISCUSSION

This study was undertaken to evaluate whether suppression of estrogen synthesis by the P450 aromatase inhibitor letrozole in boys with delayed puberty delays maturation of the growth plates, ultimately resulting in increased adult height. The rationale for giving the boys this new P450-aromatase inhibitor was the hypothesis that this treatment would help them to achieve their genetic height potential.

At the start of follow-up, the mean testosterone concentrations were approximately 10 nmol/l, suggesting that some boys were already at early or midpuberty. However, because blood samples were drawn only between 07:30 and 10:15, these levels do not reflect the mean diurnal testosterone concentrations, which are much lower (Boyar et al. 1974; Parker et al. 1975). Testosterone secretion in boys during puberty has a clear diurnal rhythm, with a peak early in the morning and significantly lower concentrations in the afternoon and evening (Boyar et al. 1974; Parker et al. 1975). In some boys, puberty might have advanced spontaneously in a short time without any treatment. However, treating these boys was justified since none had had a pubertal increase in growth velocity prior to treatment and all of those treated desired medical intervention.

LETROZOLE IN PUBERTAL BOYS

An aromatase inhibitor was used in this study for the first time in the treatment of boys with delayed puberty. This was also the first time that a potent, fourth-generation aromatase inhibitor was used in early and midpubertal adolescents.

Efficacy in inhibiting estrogen synthesis

Letrozole at a dose of 2.5 mg/day was found to be an effective inhibitor of estrogen synthesis in pubertal boys, consistent with previous reports in adult males (Trunet et al. 1993). Exogenous letrozole inhibited both the increase in 17β-estradiol concentrations associated with testosterone administration and the increase in endogenous 17β-estradiol concentrations during spontaneous progression of puberty.

Tolerability and safety

Letrozole was well tolerated. During the one-year treatment with this P450 aromatase inhibitor no side-effects indicating discontinuation of treatment were observed. However, some disadvantageous effects on BMD and HDL-cholesterol may occur. For this reason, it is important to follow up bone metabolism, BMD, and lipid concentrations during treatment with P450 aromatase inhibitors.

Estradiol has been shown to act as a germ cell survival factor in the human testis *in vitro* (Pentikäinen et al. 2000). The role of estrogen in the regulation of human spermatogenesis *in vivo* can be assessed by the findings in men with a mutation in the gene for ERα (Smith et al. 1994) or in the P450 aromatase enzyme (Carani et al. 1997). The man with a mutation in the ERα gene had a testis volume of 20-25 ml and a normal sperm density (25 x 10^6 / ml) but a decreased sperm viability of 18% (normal >50%) (Smith et al. 1994). The aromatase-deficient male had a subnormal testis volume (8 ml) and a decreased sperm count (≤1 x 10^6 / ml; normal >20 x 10^6 / ml) with 100% immotile spermatozoa (Carani et al. 1997). However, abnormal findings in semen analysis of the aromatase-deficient subject may be unrelated to suppression of estrogen action since azoospermia and infertility were also reported in the subject's brother, who had a normal P450 aromatase gene (Carani et al. 1997). Moreover, the results of semen analysis did not change during treatment with transdermal estradiol (Carani et al. 1997), suggesting nonestrogen-dependent spermatogenic damage. Neither treatment with testosterone and letrozole nor treatment with testosterone alone had an adverse effect on testis size or inhibin B concentration. These findings suggest that a one-year treatment with P450 aromatase inhibitors in early and midpubertal boys does not adversely affect spermatogenesis. Sperm analysis will ultimately confirm this issue.

EFFECT OF P450 AROMATASE INHIBITION

Growth

The boys treated with testosterone and letrozole grew at a slower rate during the first 5 months of treatment than those treated with testosterone alone. This finding confirms testosterone accelerating growth via an estrogen-dependent mechanism. The growth-enhancing effect of estrogens may result, at least partly, from stimulation of GH secretion by estrogens (Metzger et al. 1994). Consistent with this, IGF-I and IGFBP-3 concentrations increased during treatment with testosterone alone but did not change during combined treatment with testosterone and letrozole. Our findings further suggest

that other factors, in addition to estrogens, are involved in pubertal growth acceleration in males since during letrozole treatment the majority of boys grew with normal pubertal growth velocity despite low 17β-estradiol concentrations. If aromatization of androgens is inhibited, steroid biosynthesis is directed to produce 5α-DHT, and subsequently, 3β-androstanediol. The latter is a weak estrogen that has been demonstrated to bind to estrogen receptors (Kuiper et al. 1997) and may therefore have estrogenic effects. Thus, the normal pubertal growth velocity during letrozole treatment could also have resulted from activation of ER.

Consistent with the original hypothesis, inhibition of estrogen synthesis did delay bone maturation. It is noteworthy that in boys treated with both testosterone and letrozole bone maturation was slower, despite considerably higher androgen concentrations, than in those treated with testosterone alone. This finding confirms the view that estrogens are more important than androgens in bone maturation in pubertal males and agrees with observations on males who lack estrogen action (Smith et al. 1994; Morishima et al. 1995; Carani et al. 1997). Furthermore, even after discontinuation of all treatments, the progression of bone maturation was slower in the boys treated with testosterone and letrozole than in those treated with testosterone alone, indicating that the effect of treatment outlasts the treatment period (Joss et al. 1989).

Delayed bone maturation simultaneously with good growth response resulted in an increase in predicted adult height in boys treated with testosterone and letrozole. This supports the primary hypothesis that inhibition of estrogen synthesis in growing adolescents increases adult height. Since boys with CDP do not appear to fully exploit their genetic growth potential (Crowne et al. 1990; LaFranchi et al. 1991; Albanese et al. 1993, 1995), it is possible that these boys could achieve an adult height closer to their full potential if estrogen actions are inhibited. The observations that the predicted adult height did not change either in the boys who were treated with testosterone alone or in those who had received no treatment are consistent with previous studies, which have shown that androgen treatment does not increase adult height (Blethen et al. 1984; Martin et al. 1986; Albanese et al. 1993). When adult height predictions by the Bayley-Pinneau method were calculated, the table for boys with average skeletal maturity had to be used as the bone ages in most of the boys exceeded the range of bone ages reported for boys with retarded skeletal maturity. One might speculate whether this had biased the results. Adult height prediction was, however, only a tool to illustrate whether aromatase inhibition delays bone maturation. This tool has limitations and biases, and whether one-year letrozole treatment in pubertal boys actually increases adult height will only be confirmed when these boys reach adulthood.

The two treatments advanced the appearance of secondary sexual characteristics similarly, despite considerably higher androgen concentrations in the boys treated with

testosterone and letrozole. The reason why highly supraphysiological androgen concentrations did not advance puberty more rapidly is unclear. Possibly, the maximal effect at cell level is attained with a certain concentration of testosterone, and concentrations above this threshold level do not enhance the biological effect. Small differences in rates of progression of puberty between the two treatment groups may also have remained undetected due to the small number of patients in this study.

Peak bone mass

No significant differences were present in changes in BMC, BMD, or BMAD of lumbar spine or proximal femur between the groups treated with testosterone and letrozole and with testosterone alone. This suggests that one-year letrozole treatment in pubertal boys is unlikely to have any major harmful effect on BMD. This is supported by findings in bone turnover markers. They do not suggest a significant imbalance between bone resorption and formation during either of the treatments. The reports of males with an inactive ERα (Smith et al. 1994) or a defective aromatase enzyme (Morishima et al. 1995; Carani et al. 1997) demonstrate that estrogen action is needed for optimal development of peak bone mass in males. Patients with androgen insensitivity syndrome also have decreased BMD even before the age when peak bone mass is achieved (Bertelloni et al. 1998b; Marcus et al. 2000), indicating that endogenous androgens are also essential in the development of peak bone mass. In the letrozole-treated boys, high androgen concentrations may have to some extent compensated for possible harmful effects of low estrogens. However, one must recognize the limited power of a study of this size, particularly in detecting minor or rare side-effects. Confirmation of the safety of this treatment requires a larger study sample. It is noteworthy that no statistically significant increase occurred in femoral neck BMD or lumbar spine BMAD during suppression of estrogen action by letrozole, while these parameters showed an increase in the boys treated with testosterone alone who had intact P450 aromatase activity.

Some evidence exists that estrogens are more important than androgens in the regulation of developing peak bone mass in males. The rate of increase in BMD of the arms of young men correlated positively with estrogen but not testosterone concentrations (Khosla et al. 2001). While a positive correlation between the changes in lumbar spine BMAD and 17β-estradiol was found, no association was present between the changes in BMAD and testosterone or DHT. If disadvantageous effects on bone mass accretion occur during letrozole treatment, they may be also due to lower activity of the GH-IGF-I axis. GH has a significant role in bone mass accretion during growth.

In children with GH deficiency, reduced BMD has been demonstrated to increase during GH treatment (Zamboni et al. 1991; Saggese et al. 1993; Boot et al. 1997; Baroncelli et al. 1998). During letrozole treatment IGF-I and IGFBP-3 concentrations remained unchanged, while during treatment with testosterone and placebo both concentrations increased. Moreover, changes in lumbar spine BMAD correlated positively with IGF-I concentrations, and changes in femoral neck BMAD with IGFBP-3 concentrations. Since estrogens have a very important role in the regulation of BMD directly or indirectly through the GH-IGF-I axis (Metzger et al. 1994), it is essential to follow up BMD regularly during treatment with P450 aromatase inhibitors.

Although the areal BMD in the femoral neck increased in the boys treated with testosterone alone, the BMAD, an estimate of volumetric BMD, decreased within the first 5 months, which was unexpected. This may have resulted from an inaccuracy in estimated BMAD since the model for calculating BMAD had not been validated by a direct measurement of the volume of the femoral neck (Katzman et al. 1991). However, the decreasing trend in the femoral neck BMAD did not reach statistical significance at 12 or 18 months in any of the groups, which is in accord with previous findings of unchanged values of femoral neck BMAD in boys with increasing age (Kröger et al. 1992; Faulkner et al. 1996).

It is unclear whether the timing of puberty is a significant determinant of peak BMD in men. Men with a history of CDP have been shown to have osteopenia in adulthood (Finkelstein et al. 1992, 1996, 1999). On the other hand, normal volumetric BMD has also been reported in these men (Bertelloni et al. 1998a). Reasons for the discrepancy between these studies remain uncertain, although they may be related to most of the men in the latter study receiving androgen therapy to induce puberty (Bertelloni et al. 1998a). However, the possibility that a history of delayed puberty may be associated with osteopenia in adult life further highlights the importance of a close follow-up of developing bone mass during treatment with P450 aromatase inhibitors in boys with delayed puberty.

Serum lipid concentrations

HDL-cholesterol concentration decreased in the boys treated with testosterone and letrozole, but did not change in those treated with testosterone alone. This suggests that letrozole may have disadvantageous effects on HDL-cholesterol metabolism. The finding agrees with observations on aromatase-deficient males whose subnormal HDL-cholesterol concentrations increased during estrogen administration (Morishima et al. 1995; Carani et al. 1997; Bilezikian et al. 1998). In the letrozole-treated group, the

lowest level in HDL-cholesterol concentration was observed at 5 months, and no decrease was found thereafter despite letrozole treatment continuing. Moreover, the HDL-cholesterol concentration in the letrozole-treated boys was similar to levels in the boys of the other two groups 6 months after discontinuation of all treatments. Thus, letrozole treatment for one year in pubertal boys is unlikely to have a permanent harmful effect on HDL-cholesterol. However, the findings emphasize the importance of regular follow-up of HDL-cholesterol during administration of P450 aromatase inhibitors.

The more profound decrease in HDL-cholesterol concentration in the group treated with testosterone and letrozole than in the group treated with testosterone alone may be due to the greater increase in androgen concentrations with the former treatment. This is supported by the finding that decreasing HDL-cholesterol concentrations correlated with increasing testosterone concentrations. Previous studies have also suggested that androgens regulate HDL-cholesterol in boys during puberty (Kirkland et al. 1987; Arslanian et al. 1997; Morrison et al. 1998, 2000; Saad et al. 2001). On the other hand, the decrease in HDL-cholesterol concentration in the boys treated with testosterone and letrozole may in part have resulted from the inhibition of estrogen action. Estrogens suppress the activity of hepatic lipase (Applebaum et al. 1977) which catalyzes the degradation of HDL_2 lipids. However, no correlation was found between changes in HDL-cholesterol concentrations and changes in 17β-estradiol concentrations in this study. Neither was there any association between estradiol and HDL-cholesterol concentrations in earlier studies of adolescent boys (Morrison et al. 1998, 2000). Thus, estrogens may have a less important role than androgens in the regulation of HDL-cholesterol in boys during early and midpuberty. The difference in changes in HDL-cholesterol in the two treated groups may also partly have been due to differences in changes in BMIs. BMI increased concomitantly with decreasing concentrations of HDL-cholesterol during the treatment with testosterone and letrozole, but neither BMI nor HDL-cholesterol changed in the group treated with testosterone alone. Moreover, decreasing HDL-cholesterol concentrations correlated with increasing BMI. The previous findings of an inverse relationship between HDL-cholesterol concentration and BMI in adolescent boys (Morrison et al. 1998, 2000) supports the importance of body composition in the regulation of HDL-cholesterol concentration.

The concentrations of LDL-cholesterol and triglycerides did not change during either of the treatments, suggesting that treatment with a P450 aromatase inhibitor does not contribute significantly to the regulation of concentrations of LDL-cholesterol or triglycerides in early and midpubertal boys. It is noteworthy that no changes occurred in concentrations of LDL-cholesterol and triglycerides in either treatment group despite great differences in sex steroid concentrations. This suggests that sex steroids do not

have important roles in the regulation of LDL-cholesterol and triglycerides in boys at this stage of puberty. However, previous studies have reported that changes in lipoprotein concentrations in adolescent boys can be explained, to some extent, by changes in body mass, in testosterone and estrogen levels, and their interactions (Laskarzewski et al. 1983a, 1983b). Moreover, estrogens at physiological concentrations appear to have a favourable effect on LDL-cholesterol and triglycerides in adult males (Morishima et al. 1995; Carani et al. 1997; Bilezikian et al. 1998). For these reasons, follow-up of lipid profiles during administration of P450 aromatase inhibitors is recommended.

Insulin sensitivity

Decreasing insulin sensitivity during puberty simultaneously with the rise in concentrations of sex steroids is a well-known phenomenon (Amiel et al. 1986, 1991; Bloch et al. 1987; Caprio et al. 1989; Smith et al. 1989). The role of sex steroids in the development of puberty-associated insulin resistance is, however, unclear.

P450 aromatase inhibition in early and midpubertal boys does not appear to have disadvantageous effects on insulin sensitivity. During letrozole treatment fasting insulin concentrations decreased, suggesting an improvement in insulin sensitivity despite higher increases in androgen concentrations than in the group treated with testosterone alone, in which fasting insulin concentrations remained unchanged. This finding implies that an increase in the concentration of androgens during puberty does not directly contribute to the development of puberty-associated insulin resistance in boys, agreeing with the results of other studies (Arslanian et al. 1997; Saad et al. 2001). Neither do estrogens appear to have a direct role in the regulation of insulin sensitivity in boys during puberty. The changes in 17β-estradiol concentrations did not correlate with changes in fasting insulin concentrations. The changes in insulin sensitivity in boys during progression of puberty also showed no association with changes in estradiol concentrations in another study (Goran et al. 2001). Decreasing insulin sensitivity has been demonstrated to be associated with increasing BMI in adolescent boys (Cook et al. 1993). The decrease in the fasting insulin concentration during letrozole treatment was obviously not a consequence of decreased body mass since BMI increased during treatment. The decrease may have been due to the suppression of GH secretion, as no pubertal increases in IGF-I or IGFBP-3 concentrations were observed during letrozole treatment. This assumption is supported by the finding that changes in fasting insulin concentrations correlated positively with changes in IGF-I concentrations. These observations are in accord with a decrease in insulin sensitivity during puberty resulting

from the increasing action of GH (Amiel et al. 1986; Bloch et al. 1987; Caprio et al. 1989; Smith et al. 1989; Heptulla et al. 1997; Moran et al. 2002). Since androgens can be converted to estrogens which are able to augment GH secretion (Metzger et al. 1994), the effects of GH on regulation of insulin sensitivity may also indirectly reflect the action of sex steroids.

Fasting insulin concentration, which was used as an index of insulin resistance, has been demonstrated to correlate well with insulin resistance measured by the euglycemic insulin clamp technique (Olefsky et al. 1973). While measuring only a single fasting insulin concentration is not optimal for assessing insulin sensitivity, more accurate methods, such as the euglycemic insulin clamp techinique, would have made the study protocol too demanding for the boys. Differences in fasting insulin concentrations between the two treated groups could theoretically be explained by differences in blood glucose concentrations, but this is considered unlikely since letrozole has not been shown to affect fasting blood glucose (Berstein et al. 2002). However, absence of fasting blood glucose concentrations impedes interpretation of results. Further studies with more appropriate methods are needed to confirm whether P450 aromatase inhibitor treatment has an impact on insulin sensitivity.

ROLE OF SEX STEROIDS IN GONADOTROPIN SECRETION IN PUBERTAL BOYS

Endogenous androgens appear to inhibit gonadotropin secretion in boys from the early stages of puberty onwards (Santen et al. 1976). Supraphysiological estradiol concentrations, attained by estradiol infusion, decreased LH concentrations in early and midpubertal boys (Kletter et al. 1997), which suggests the existence of negative feedback between estrogen and gonadotropin secretion. This study showed that when the action of low concentrations of endogenous estrogens was suppressed by letrozole, LH and FSH concentrations increased despite very high androgen concentrations. This indicates that the negative feedback between endogenous estrogens and gonadotropin secretion, established in adult men (Smith et al. 1994; Morishima et al. 1995; Carani et al. 1997; Bilezikian et al. 1998; Hayes et al. 2000), is operative from early puberty onwards. Furthermore, the observations suggest that androgens have a less important role than estrogens in the regulation of LH and FSH secretion in early and midpubertal boys.

When the action of endogenous estrogens was suppressed by the treatment with testosterone and letrozole, the LH pulse amplitude and the GnRH-induced LH response increased, but the LH pulse frequency, which is assumed to reflect the frequency of

hypothalamic GnRH secretion (Clarke et al. 1982; Levine et al. 1982), was unaffected. These observations suggest that low concentrations of endogenous estrogens in early and midpubertal boys may not influence the GnRH pulse generator, and that, in boys during early and midpuberty, the site of action of estrogens is the pituitary. Previous findings have, however, been contradictory. In early and midpubertal boys, estradiol infusion decreased LH concentrations and the LH pulse frequency but had no effect on the GnRH-induced LH response or the LH pulse amplitude (Kletter et al. 1997), suggesting a hypothalamic site of action for estrogens. The reason for this discrepancy is unclear. One possibility is that supraphysiological concentrations of estrogens and endogenous estrogens act on gonadotropin secretion differently. In the previous study, supraphysiologic estradiol concentrations were attained by exogenous administration (Kletter et al. 1997). By contrast, in the group treated with testosterone plus letrozole in this study, the effects of suppression of low, early pubertal concentrations of estrogens were demonstrated. The evaluation of the precise sites of the negative feedback of gonadal steroids in the hypophyseal-pituitary unit is difficult *in vivo*. In humans, monitoring hypothalamic GnRH secretion directly is not possible since GnRH is consumed in the hypophyseal-portal blood due to its short half-life of 2-4 minutes (Hayes et al. 1998). In animal models, an exact temporal relationship between GnRH secretion and LH secretion has been observed (Clarke et al. 1982; Levine et al. 1982), and thus, monitoring LH pulse frequency in humans has been used in estimating the frequency of hypothalamic GnRH secretion. The results of indirect methods may not, however, demonstrate the true site of action at the hypothalamic-pituitary unit. This, as well as different pulse detection programs which were used, may also partly explain the discrepancy between this and the previous study (Kletter et al. 1997). Thus, future studies with larger patient groups and longer periods of blood sampling are needed to confirm the conclusions drawn here regarding the pituitary site of action of endogenous estrogens and to clarify whether endogenous estrogens also act at the hypothalamus in boys during early and midpuberty.

The negative feedback regulation between FSH and endogenous estrogens has previously been observed in adult males (Trunet et al. 1993; Hayes et al. 2000). The results of this study suggest that this regulatory loop is already operative in early and midpubertal boys. Although inhibin B participates in the regulation of FSH secretion from early to midpuberty onwards (Nachtigall et al. 1996; Andersson et al. 1997; Byrd et al. 1998; Raivio et al. 2000; Hayes et al. 2001), the increase in FSH concentrations during letrozole treatment was probably not due to a diminished negative feedback signal from inhibin B, for inhibin B concentrations increased concomitantly with FSH concentrations.

The nocturnal augmentation of gonadotropin secretion, which is characteristic of early and midpubertal boys (Boyar et al. 1972; Beck et al. 1980), was demonstrated before the start of treatments in all of the boys and also in all boys during the testosterone plus letrozole treatment. Thus, the suppression of estrogen action does not affect the diurnal profile of gonadotropin secretion, nor does the considerable increase in the concentrations of androgens. This observation is in accord with previous findings of a diurnal rhythm in gonadotropin secretion in children with gonadal dysgenesis (Boyar et al. 1973; Ross et al. 1983) and indicates that the circadian rhythm of gonadotropin secretion is regulated by mechanisms mediated by the central nervous system.

FUTURE PROSPECTS

These results suggest that an increase in adult height can be attained in growing adolescent boys by inhibition of estrogen action. However, at the time of completion of this study, the skeleton was relatively immature and growth was not decelerating in most of the boys. Therefore, it is important to confirm whether a one-year P450 aromatase inhibitor treatment actually increases adult height by following up the boys until attainment of final adult heights. Although our findings suggest that treatment for one year with letrozole in pubertal boys is unlikely to have any major harmful effects on developing peak bone mass, only by measuring BMD after the attainment of peak bone mass can firm conclusions be drawn. Moreover, while the treatment had no adverse effects on testis size or inhibin B concentration, suggesting that it did not adversely affect maturing spermatogenesis, sperm analysis is needed to ultimately confirm this issue.

Since the results showed that bone maturation can be delayed by suppressing the action of estrogens in growing adolescents, fourth-generation P450 aromatase inhibitors may prove to be an efficient treatment in various growth disorders. Future studies are required to establish whether treatment with aromatase inhibitors can be used, for instance, in patients with precocious puberty or congenital adrenal hyperplasia with significantly advanced bone age, or in healthy boys with genetic short stature.

SUMMARY AND CONCLUSIONS

This prospective, randomized, placebo-controlled study was undertaken to evaluate whether suppression of estrogen synthesis in boys with delayed puberty delays bone maturation and ultimately results in increased adult height. Since some boys with CDP do not exploit their full genetic growth potential, we hypothesized that these boys would achieve an adult height closer to their genetic growth potential if estrogen actions were inhibited.

A total of 33 boys with CDP participated. Ten boys, who decided to wait for spontaneous progression of puberty without medical intervention, composed the untreated group. Twenty-three boys desired medical intervention and were randomized to receive one of two treatments: 12 boys received testosterone and placebo, and 11 boys testosterone and a specific and potent P450 aromatase inhibitor, letrozole, for suppression of estrogen synthesis.

The main conclusions of this study are:

1. Letrozole was well tolerated and effectively inhibited estrogen synthesis in adolescent boys. Estradiol concentrations remained at pretreatment level during the administration of letrozole, whereas the concentrations increased during treatment with testosterone alone and during spontaneous progression of puberty.

2. Letrozole treatment delayed bone maturation. During the 18-month follow-up bone age advanced 0.9 year in the group treated with testosterone and letrozole and 1.7 years in the group treated with testosterone alone. An increase of 5.1 cm in predicted adult height was seen in the boys who had received testosterone and letrozole, but no change was seen in those receiving testosterone alone or in the untreated boys. This finding suggests that an increase in adult height can be attained in growing adolescent boys by inhibiting estrogen action, allowing the boys with CDP to achieve an adult height closer to their genetic growth potential.

3. No significant differences in changes in BMC, BMD, or BMAD, an estimate of true volumetric BMD, were observed between the two treated groups. Nor were there any significant imbalances between bone resorption and formation in either of these groups. These findings suggest that a one-year letrozole treatment in pubertal boys is unlikely to have any major harmful effects on BMD. However, although lumbar spine BMAD increased in both treated groups, in the group treated with testosterone and letrozole, the

increase was statistically significant for only 6 months after discontinuation of letrozole treatment. Thus, close follow-up of BMD during treatment with P450 aromatase inhibitors is warranted.

4. HDL-cholesterol concentration decreased in the boys who had received testosterone and letrozole, whereas no change was observed in those receiving testosterone alone. However, the HDL-cholesterol concentrations were similar in all groups 6 months after the discontinuation of treatments. No changes in the concentrations of LDL-cholesterol or triglycerides were observed in any of the groups. Because treatment with P450 aromatase inhibitors may have disadvantageous effects on HDL-cholesterol metabolism, a follow-up of lipid profile is important.

5. Fasting insulin concentrations decreased during letrozole treatment, but no change was observed during treatment with testosterone alone, suggesting that treatment with P450 aromatase inhibitors in early and midpubertal boys has no disadvantageous effects on insulin sensitivity. Insulin concentration remaining unchanged in the boys treated with testosterone alone but decreasing during letrozole treatment despite a higher increase in androgen concentrations suggests that rising androgen concentrations during puberty do not directly contribute to the development of puberty-associated insulin resistance in boys.

6. During the treatment with testosterone alone and simultaneously with increases in testosterone and 17β-estradiol concentrations basal LH and FSH concentrations decreased, but GnRH-stimulated gonadotropin responses remained unchanged. During the treatment with testosterone and letrozole, concomitantly with a higher increase in testosterone concentration and unchanged concentrations of 17β-estradiol, an increase occurred in basal gonadotropin concentrations, GnRH-induced LH response, and LH pulse amplitude. These findings demonstrate that low physiological concentrations of endogenous estrogens inhibit LH and FSH secretion in boys during early and midpuberty. The results also suggest that androgens have a less important role than estrogens in the regulation of gonadotropin secretion in these boys. Moreover, during this stage of puberty the endogenous estrogen-mediated regulation of LH secretion apparently occurs at the site of the pituitary.

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